Training and fucose metabolism in chick brain

Thesis

TRAINING AND FUCOSE METABOLISM

IN

CHICK BRAIN

by

Norah R. McCabe B.Sc.

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Table of Contents

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Abbreviations.

A.A.	Amino-acids
Ad	archistriatum dorsale
Aniso	anisomycin
Av	archistriatum ventrale
B.D.T.	Brightness Discrimination Task
Bq	Becqurel
Cardiac Cond.	Cardiac Conditioning
Cb	cerebellum
CX.	cycloheximide
DNA	deoxribonucleic acid
E	ecostriatum
Electrosch.	electroschock
E.C.F.	extracellular fluid
Extern.Med.	external medium
EGTA	ethyleneglycol-tetra-acetic acid
Hp.	hippocampus
Homog	homogenate
HD	hyperstriatum dorsale
hr	hour
HV	hyperstriatum ventrale
HVdv	hyperstriatum ventrale dorso-ventrale
HVvv	hyperstriatum ventrale ventro-ventrale
K	kilodalton
LPO	lopus parolfactorius
LTP	long term potentiation
(M)	methylanthranilate

MHV	medial hyperstriatum ventrale
min	minute
ml	millilitre
N	neostriatum
NB	nucleus basalis
NC	neostriatum caudale
PA	paleostriatum augmentatum
P.A.L.	passive avoidance learning
pg	picogram
PP	paleostriatum primitivium
RNA	ribonucleic acid
S	saline
T.C.A.	trichloroacetic acid
(W)	water

List of Figures and Tables

Abstract

This thesis reports an extensive study on the changes in fucose metabolism when chicks are trained on a passive avoidance learning paradigm. The study is based on the analysis of an observed increase in fucose incorporation observed *in vivo* (Sukumar *et al* 1980). Experiments were conducted to determine the mechanism of this increase and an *in vitro* fucosylation system was established to isolate the specific glycoproteins involved. Finally a series of experiments were performed to investigate the exact nature of this training-related increase in fucosylation by interventive methodology.

Chapter 1 introduces the extensive subject of learning and memory mainly from a physiological view, touching briefly on some of the major psychological classifications.

Chapter 2 is a review of the physiological literature on learning and memory in the chick, covering two behavioural paradigms: imprinting and a passive avoidance learning task. It covers the basic anatomy and function of the avian telencephalon, and finally introduces the topic of glycoprotein structure and function.

Chapter 3 is the first experimental Chapter, and covers the analysis of one of the enzymes in the metabolic pathway of fucose incorporation into glycoproteins: fucokinase. This Chapter documents the assay procedure, the physiological conditions for maximum enzyme activity and finally the effects of training on fucokinase activity. Activity is not regulated by increases in the physiological concentration of calcium or c'AMP or c' GMP. Maximum activity is elicited with a specific concentration of magnesium, 3.3mM. One hour

and six hours after training there is a significant increase in fucokinase activity, which is located to the left base and right forebrain roof respectively. An extensive discussion of the experimental results and hypotheses are presented.

Chapter 4 covers the experimental procedure of obtaining an active fucosylation system in chick forebrain slices *in vitro*. When chick forebrain slices are cut on a McIlwain tissue chopper to a thickness of 0.4 mm, and incubated in a Hepes buffer pH 7.4 for 3 hr at 42 C, in the presence of labelled fucose an active fucosylation system is maintained. Fucose incorporation rates as high as 37 nmole/g prot/hr were observed. Slices of forebrain tissue from (M) trained and (W) control chicks were analysed for fucose incorporation *in vitro* at specific times after training. The increase in fucose incorporation after training which was observed *in vivo* (Sukumar *et al* 1980) was replicated in *in vitro*. The increase was localized to the right forebrain base. On subcellular fractionation the increase in fucose incorporation was found in the P3 fraction. An extensive study using gel electrophoresis of the P3 fraction indicated that there was greater labelling of proteins with molecular weights 120K - 82K in the trained chicks compared to controls. The experimental findings are discussed in relation to previous *in-vivo* work.

Chapter 5 is concerned with the question of the exact nature of the training-related increase in fucose incorporation. Experiments were devised to determine if the increase was an independent increase in fucose incorporation or, alternatively, if it was associated with an increase in protein synthesis *de novo*. A series of four experiments are outlined analysing both the effects of cycloheximide and saline on fucose and leucine incorporation *in vivo* and *in vitro* in

trained and control chicks. The training-related increase in fucose incorporation was abolished when cycloheximide was injected intracranially, which would suggest that the increase in fucosylation was occurring on protein synthesis de novo. The results of these experiments are discussed in relation to the time after training during which protein synthesis is important in memory formation.

Chapter 6 is a general discussion of all the experiments and attempts to substantiate and rationalize a link between all the observed results.

Chapter 7 covers a number of experiments for future work.

Publications and Presentations

The experiments reported in Chapter 4 have been published as a paper in Neurochemical Research (1985).

Experiments reported in Chapter 4 and 5 have been presented as a poster at The 10th International Meeting for Neurochemistry, Italy 1985.

The major findings of the work outlined in this thesis have been orally presented at The Neurochemical Open Meeting, The Biochemical Society, Oxford 1985.

CHAPTER 1

A REVIEW OF LEARNING AND MEMORY

Biologists have approached the study of learning and memory from many viewpoints in order to answer the basic questions about them (why, what, how and where?) from both physiological and psychological perspectives. In this review, I wish to discuss from the former view what learning and memory are, touching briefly on some of the classical psychological classifications of memory, and finally to examine work on neurobiological aspects.

A very general definition of learning may be; learning has occurred if one observes an adaptive change in an animal's behaviour that can be attributed to changes in a particular set of contingent circumstances. A translation of this definition into physiological terminology would be; learning is a process in which neuronal information pathways between entry and exit points in the central nervous system are modified as a result of a previous experience.

1.1 PSYCHOLOGICAL ASPECTS OF LEARNING

The first biologist to address the issue of learning was the Russian digestive physiologist Pavlov. He observed that an alimentary reflex in dogs, salivation, could be elicited by a stimulus other that food. After a number of trials of simultaneous 'food - stimulus

presentation, the stimulus alone was able to elicit salivation. This form of learning, known as classical conditioning, was the first example of behavioural modification to be described (Pavlov 1927).

The work of Pavlov was unknown in the West when the American psychologist Thorndike was independently studying learning patterns in animals. After observing the way in which kittens escaped from a puzzle box (a cage from which they can escape by moving a lever), Thorndike (1911) proposed that learning occurs through the consequences of behaviour. He formalised this idea in 'The law of Effect'; 'when an association is made between a situation and a response that is accompanied or followed by a satisfying state of affairs, the strength of the association is increased. When it is followed by an annoying state of affairs, its strength is decreased'. This type of learning differs from that of classical conditioning; for in Thorndike's hypothesis, it is the consequence of the response that is important in forming or abolishing stimulus - response associations (Thorndike 1911). In classical conditioning, an association is formed regardless of the nature of the response as long as two stimuli occur closely together in time.

In the late 1920s, the American psychologist Skinner was attempting to devise a system of classification that would lead to the identification of 'units of behaviour'. However, he felt that classical conditioning, describing behaviour in terms of reflexes, did not explain the occurrence of a whole range of behaviours. Following Thorndike, Skinner proposed a category of behaviour called 'operant' behaviour. He suggested that animals are constantly emitting behaviours, or operants, and that whether operants are incorporated into the total behavioural repertoire of the animal depends on the consequences of performing the operants. Confining rats to his own

design of puzzle box, the Skinner box, Skinner was responsible for the development of a type of learning that is now called 'instrumental' or 'operant' conditioning (Skinner 1950).

Essentially, Pavlov and Skinner described different conditions under which learning can occur; in classical conditioning the animal learns via the association of two stimuli, one of which predicts the other. In operant conditioning, it is the consequence of a response that results in an association between a stimulus and that response. In general, the stability of operant conditioning is much greater than that of classical conditioning. If stimuli are not presented simultaneously and sufficiently often, then classical conditioning breaks down; in contrast, operants can be very resistant to extinction.

These two paradigms of learning, classical and operant conditioning, have gained great theoretical importance in studies of the experimental psychology of animal learning. However. there are many varieties of learning that do not fit easily within these two paradigms; for example, imprinting is a form of learning that occurs in the early stages of an animal's life by which social preferences are established (discussed in Chapter 2). In the literature many different learning tasks have been presented to laboratory animals by researchers (see Table 1.1), and are often described as specific, separate learning paradigms. But most, if not all, can be considered basically as types of operant conditioning. In all of these paradigms, it is the consequence of repeating a particular operant that becomes associated with some characteristic to be learnt (for example, obtaining a reinforcement by pressing a bar in a Skinner box).

Learning, if it has led to a change in consequent behaviour encompasses three major processes:

1. acquisition: the phase of acquiring information

2. memory: storage and consolidation

3. retrieval: the recall of information

1.2 PSYCHOLOGICAL ASPECTS OF MEMORY

When an animal has 'learned' something (for example, the ability to perform a task), then it has acquired information, stored it and subsequently has the ability to show recall of this information. However, not all learning leads to these three processes - if there is an inability to recall, then one assumes a memory or a memory trace has not been formed, or indeed that the trace has diminished with time. Memory refers to a capacity to code or register a modification that occurs in the nervous system as a result of learning. Psychologists are mainly concerned with the performance characteristics of memory (what type of memory is being elicited), so that recall for a particular task is evident. Memory for a particular learned task can only be elicited by performance recall, however memory called upon enabling the animal to elicit recall may be varied - for example, a response that was learned when the animal was hungry may not be exhibited when the animal is satiated. This does not mean that the memory was not formed, rather, it is not required for immediate behaviour. I do not intend to consider the rather complex inter-relations between learning and motivation in this thesis.

Let us first consider briefly the vast literature that exists on the psychological classifications of memory. These are based on the hypothesis that memory is composed of functionally separate systems, which are used for the recall of particular sequences of behaviour. The units that are learned and recalled by a subject do not always correspond to the units present for learning. Cognitive processing will affect the number and type of memory units formed, and subsequently recalled by individual subjects (Tulving 1972). Much of this research has been conducted on human volunteers and patients with specific brain injuries, the latter have the ability to show recall for certain learned tasks, or specific aspects of the task, but an inability to recall other learned tasks.

Memory theorists working on humans have found it profitable to divide long-term verbal memory into two components: episodic and semantic memory (Tulving 1972). Episodic memory is defined as memory for specific personally experienced events, while semantic memory is defined as memory for general principles and associations.

Another proposed distinction between memory systems is the distinction between reference memory and working memory, which was developed from studies on experimentally lesioned animals (Olton et al 1979). Reference memory is a system claimed to be involved in learning those aspects of a task that are constant from trial to trial (where there is no modification between trials), while working memory is defined as a more flexible system for shorter-term storage of aspects of a task that do change from trial- to- trial.

Yet another classification which delineates different memory types comes from the literature on artificial intelligence (Anderson 1982), involving procedural and declarative memory. The former results from the learned process, which is put into operation for a particular recall process, whereas declarative memory is a set of facts or data

related to the learning process, which can be retrieved directly on demand (reviewed by Squire and Butters 1984). These classifications of the different types of memory are representative of the widespread literature that is available on the various approaches to analysing memory on a purely psychological basis. The debate largely surrounds the division of mental processes that are actually called into action during a recall event. I think it is necessary to be aware that such a vast amount of related literature does exist, but I do not consider its further discussion entirely relevant to this thesis.

1.3 PHYSIOLOGICAL ASPECTS OF LEARNING

An alternative approach has been adopted by physiologists, who have concentrated on the cell-biological implications of learning and memory formation. They have sought, and the search still goes on, for the answers to the following questions. What neuronal factors are concerned with the process(es) of learning, what are the structural and functional physiological changes in the brain that are essential for the formation of a memory and where do they occur?.

As early as the 1600's, scientists proposed variations on the basic theory that learning results in the formation or strengthening of pathways in the brain. In 1661, Descartes suggested that the memory trace was associated with 'pores in the brain' which acquired a facility to be opened more easily than others. More recently, Cajal (1911) suggested that the relevant physiological mechanisms were associated with changes in neuronal connections. Further extrapolation of these theories led to the phenomenon of neuronal plasticity; modification of the neuronal network, specifically associated with changes in synaptic connectivity. Armed with these conceptions, neurobiologists have devoted their research to answering

the following questions; what are the necessary, sufficient and exclusive physiological mechanisms which occur in the brain to induce specific alterations in synaptic connectivity due to a learning experience? Before embarking on such research Rose (1981) has suggested a number of criteria which must be satisfied by related experiments before any correlations can confidently be drawn between a physiological change and a behavioural response. These include the specificity of change to a neuroanatomical locus, the time course of change to the development of memory and the abolition of the detected change with amnesia.

If learning does give rise to changed connectivity in neural circuits, what sort of a change is it and where does it occur?. In trying to answer this question, neurobiologists have adopted many different approaches, and thus introduced a major source of diversity into this area of research. Is it a morphological change, associated with changes in neural network density or shape?, and/or biochemical changes associated with increased synthesis, degradation or turnover of certain neural molecules?, and/or an electrophysiological change, a variation in steady-state neuronal activity at specific loci? Do the physiological changes associated with learning and memory involve one, or all of these processes and do they occur in parallel or in sequence in a time dependent manner?

The application of biochemical methodology to the study of behaviour can be conveniently categorized as being either (a) correlative or (b) interventive (Agranoff et al 1978). Some researchers have concentrated on the correlative approach, taking at least two groups of animals, a control group and an experimental group which have being trained on a particular task. The brains from animals of both groups are then analyzed to determine changes at a

specific level which have occurred as a result of the training procedure (Hyden & Egyhazi 1964; Popov et al 1980, 1983; Rose et al 1980; Rose 1981).

Others have adopted an interventive approach, applying neurotoxic agents and neuronal lesions to the brain and consequently determining the effect on the animal's ability to learn and recall a learned task. This type of research reveals structures and agents which may be necessary for the acquisition, establishment and/or retrieval of the learned task (Agranoff et al 1967, 1978; Flexner & Flexner 1966, 1967, 1968).

Another line of interventive investigation is the modulation of behaviour by neurochemicals. Particular pharmacological agents (Bennett & Rosenzweig 1971) and endocrinological treatments (Clifton & Andrews 1982; Clifton et al 1983) are used to determine what factors can promote the process of acquisition (the time taken to learn the task), increase the retention time of memory and/or improve retrieval.

The second major diversification in research into the neurobiological basis of learning and memory is the experimental animal used and the paradigm investigated. Among the vertebrates, the rat (Rattus norvegicus) and chick (Gallus domesticus) have gained great popularity, while the most often used invertebrates are the molluscs ,such as Aplysia californica and Hermissenda crassicornis. Other animals used include the mouse (Mus musculus), goldfish (Carassius auratus) and the fruit-fly (Drosophila melanogaster). The choice of learning paradigm has probably led to the greatest diversification, with researchers employing a wide range of experimental paradigms ranging from innate behaviour (for example, pecking behaviour in the young chick) to the performance of specific tasks (which, as stated earlier, are basically operant conditioning tasks; an example is discrimination behaviour in the adult rat). The

list of paradigms includes passive and active avoidance, discrimination tasks, visual experience, conditioning, sensitization and habituation. A representative list is given in Table 1.1.

Probably the greatest criticism that has been levelled at some neuroscientists in this field of research is their apparent lack of thought in choosing an appropriate learning task. An animal's ability to learn about aspects of its environment is a necessary and intrinsic prerequisite for survival, and is therefore biologically relevant. For example, when a rat experiences distasteful food it shows a very distinct aversion response some time after being physically ill. The rat undergoes this form of taste aversion learning in its natural environment and the paradigm is thus biologically relevant. However, in Shashoua's (1982) experiments a goldfish is attached to a float making it swim upside down; it subsequently learns to maintain a normal posture. This task is hardly a process which a fish would encounter in natural conditions. As learning is a very important part of an animal's survival, neuroscientists interested in the physiological implications of this process should match species with learning tasks that are biologically feasible, rather than invent elaborate tasks for the animals to learn. Critics of traditional learning paradigms have advocated that a biological orientation should be considered when choosing to study a learned behaviour in a particular species, such that biological constraints, adaptive specialization and situation specificity are included; Domjan & Galef (1983) provide a clear review of this area. There are obvious limitations in choosing species and learning task which can be studied within a laboratory situation; however, given the deprivation of ecological niche, one should always consider the relevance of the task to the natural habits of the species under study.

Booth (1967) was also concerned with this great diversification in research on the cell biology of learning when he said;

"most of the effort goes into multiple parametric replications of a prematurely standardized paradigm involving virtually unexplored behavioural and physiological mechanisms."

Table 1.1 is a brief review of neurobiological research in learning and memory, showing the animal used, behavioural paradigm, parameter investigated, locus of investigation in the nervous system and reference.

Table 1.1

Paradigm	Parameter Investigated	Locus	Reference
Aplysia californica			
Conditioning	Transmitters	Cellular	Kandel & Schwartz'82
Conditioning	Ions	Cellular	Kandel *et al* '83
Sensitization	C'AMP	Cellular	Castelluci *et al* '80
Sensitization	Ca2+	Cellular	Klein & Kandel'78
Hermissenda crassicornis			
Association	Chemoreceptors	cellular	Culligan & Gelprin '73
Association	Ca2+, K+	Cellular	Alkon '79
Association	Electrical Changes	Photo-Receptors	Farley *et al* '83
Association	Voltage Changes	Cellular	West *et al* '82
Drosophoila melangaster			
Visual Discrimination	ACHase	Cellular	Folkers & Spatz'84
Odour avoidance	Memory Inhibition		Dudai'77
Odour Avoidance			Quinn & Dudai'76
Shock Avoidance	C'AMP Defeciencies		Quinn *et al* '74
Carassius auratus			
Swimming	RNA	Nucleus Cytoplasm	Shashoua'82
Swimming	Protein Synthesis	E.C.F.	Shashoua'81

Rabbit

Eyelid Conditioning	Electrical Changes	Brain	Thompson et al '73,'76

Rattus norvegicus

Passive Avoidance	Fucose Incorporation	Hippocampus	Popov et al '77
Passive Avoidance	Lesions	Striatum	Sandberg et al '84
Passive Avoidance	Cycloheximide	Cerebrum	Geller et al '69
Passive Avoidance	Peptides	C.S.F.	Mens et al '82
Passive Avoidance	Lesion	Pituitary	de Kloet et al '83
Passive Avoidance	Glycoproteins	Cerebrum	Holian et al '71
Passive Avoidance	Phosphorylation	Frontal Cortex	Morgan & Routenberg '81
Passive Avoidance	Fucose Incorporation	Neostriatum	Morgan & Routenberg'77
Active Avoidance	D.N.A. Synthesis	Cerebrum	Scaroni et al '83
Avoidance	Vasopressin		Hagan et al '82
Avoidance	Dopamine		Anisman et al '82
B.D.T.	R.N.A. Synthesis	Hippocampus	Pohle & Matthies'74
B.D.T.	Fucose Incorporation	Hippocampus	Popov et al '80
B.D.T.	Fucokinase Fucotransferase	Hippocampus	Popov et al '83
B.D.T.	Glycoproteins	Hippocampus	Popov et al '82
B.D.T.	Apomorphine	Hippocampus	Jork et al '82
Balancing	GABA Receptors	Dieters Neurones	Hyden et al '84
Handedness	Protein Synthesis	Hippocampus	Hyden & Lange'68

Maze Running	Acetylcholine	Cortex	Kreck et al '66
Conditioning	D.N.A. Synthesis	Hippocampus	Salganik et al '83
Instrumental Task	Protein Synthesis	Hippocampus	Hyden & Lange'83
Gallus domesticus			
Passive Avoidance	Fucose Incorporation Binding	Forebrain	Sukumar et al '79
Passive Avoidance	Drug Inhibition		Mark & Watts'71 Watts & Mark'70
Passive Avoidance	Fucose Incorporation	Forebrain	Burgoyne & Rose'80a
Passive Avoidance	B-Antagonist Binding		Stephenson & Andrews'81
Passive Avoidance	2-De-oxyglucose	Forebrain	Kossut & Rose'84
Passive Avoidance	Tubulin Glycoprotein	Forebrain	Mileusnic et al '80
Passive Avoidance	Morphology	M.H.V.	Stewart et al '83,84
Passive Avoidance	Fucokinase Activity	Forebrain	Loessner & Rose'83
Passive Avoidance	Leucine Incorporation	Forebrain	Schliebs et al '85 Slices press
Passive Avoidance	Fucose Incorporation	Forebrain	McCabe & Rose(in press)
Passive Avoidance	QNB Binding	Forebrain	Rose et al '80
Imprinting	Protein Synthesis	Forebrain	Longstaff & Rose'81 Midbrain
Imprinting	Uracil Incorporation	Forebrain	Bateson et al '75
Imprinting	Lysine	Forebrain Incorporation	Hambley et al '77
Imprinting	Excitatory A.A.	Forebrain	Rogers & Hambley '82
Imprinting	Lesion	H.V.	Horn & McCabe'84

Imprinting	Lesion	Forebrain	Salzen et al '75
Taste Aversion	Vasopressin		Davis et al '82
Taste Aversion	Oxytocin		Davis et al '83
Taste Aversion	Testosterone		Andrew et al '81
Taste Aversion	Gonadal Steriods		Clifton et al '85
Visual Experience	Morphology	H.A.	Bradley & Horn'79
Visual Experience	Cycloheximide.	Forebrain	Rogers & Anson'79
Visual Experience	Protein	Forebrain Synthesis	Smith et al '70
Visual Experience	Catecholamines	Forebrain	Davies et al '83

Columbia livia

Probability Learning	Lesions	H.V.	Macphail & Reilly'83
Cardiac Cond.	Lesions	Archistriatum	Cohen'75

In the invertebrates, Kandel and Schwartz (1982) and Castellucci & Kandel (1976) have extensively studied Aplysia californica, a marine mollusc which has large neurones, some up to 1000 micrometers in size. These workers have investigated electrophysiological and chemical changes consequent to sensitization, habituation and classical conditioning.

In molluscs, the mantle cavity, a respiratory chamber housing the gill, is covered by a protective sheet, the mantle shelf, which terminates in a fleshy spout, the siphon. When the siphon or mantle shelf is stimulated by a light touch, the siphon, mantle shelf and gill all contract vigorously and withdraw into the mantle cavity. This reflex can be modified by experience or training by two forms of nonassociative learning, sensitization and habituation, as well as by associative, classical conditioning.

Sensitization is a behavioural process in which an animal learns to strengthen its reflexes and to respond vigorously to a variety of previously neutral or indifferent stimuli, after it has been exposed to a noxious stimulus. Habituation is a learning process which is different from sensitization, in which an animal learns through repeated presentation to ignore a weak stimulus. Carew et al (1981) found that in addition to undergoing the latter two types of learning, Aplysia could also undergo classical conditioning. Typically in conditioning, an initially weak or ineffective conditioned stimulus becomes effective in producing a behavioural response, after it has been paired with a strong unconditioned stimulus (Pavlov 1927). This is distinguished from sensitization by the requirement for temporal pairing of the two stimuli during training.

Due to the accessibility and large size of the neurones in Aplysia, the neuronal pathways involved in these learning processes have been well mapped, and it has been suggested that the critical loci for learning processes are a set of sensory synapses which are easily identifiable. With Aplysia it has proved possible to examine the type of learning at behavioural, cell-biological, ultrastructural and molecular levels (reviewed by Kandel & Schwartz 1982).

This very elegant research has produced convincing evidence that specific molecular and electrophysiological changes do occur at the synapse as a direct result of a learned response. However, this line of research has its limitations; although invertebrates may be optimal subjects for interpretation of behavioural processes that occur at the cell-membrane level, it is not clear whether one can extrapolate these effects to those that may occur in vertebrates.

The vertebrate brain is a very large, complicated, structure with neurones ranging from 10-100 micrometers in size and in man the number approximates to 100 million; where neuronal integration is of paramount importance. Research in the development of accurate neuroanatomical atlases of vertebrate brains has been very productive and many proposed areas associated with the loci of learning and memory formation have been well documented, for example the hippocampus in the rat and the forebrain (in particular the hyperstriatum ventrale) in the chick. However, the search for the exact location of physiological change is severely limited by the density of neurones, the large number of synaptic connections and the presence of glial cells; research is now showing that the latter may have other functions than a supportive role in neuronal function. Hence, neuroscientists who work with vertebrates have indeed a major task, trying to locate specific changes in particular brain regions as

a result of a behavioural response. Morgan and Routtenberg (1977,1981), Popov et al (1976 (a) (b),1980) and Thompson et al (1983) and have chosen the rat as the experimental animal, while Bateson (1966), Horn (1981), Davis et al (1982, 1983) and Rose (1981) have chosen the chick.

Not only is there a problem with trying to locate specific physiological changes in the brains of vertebrates, but also there can be problems with the behaviour. While training the animals it is necessary to try and reduce the number of confounding variables within and between experimental groups, so that behaviour is standardized as far as possible.

I shall refer more specifically to this problem in Chapter 2 as I have found variability in animal behaviour to be a major source of difficulty in my training experiments.

As a result of these factors, the neurobiologist must make a compromise, (1) work with vertebrates and have a reduction in the specificity of the physiological change resulting from the behavioural response, or (2) work with invertebrates and have a reduction in the range and complexity of behaviour studied.

Whichever experimental animal is used, it is important that before any correlation between physiological change and a behavioural response is documented, it must meet certain criteria; the change observed must be necessary, sufficient and exclusive to the learned response.

1.4 PHYSIOLOGICAL ASPECTS OF MEMORY FORMATION

Some physiologists, rather than concerning themselves with the type of memory being formed, have choosen instead to discuss memory in terms of phases, which classifies different memory stages according to the length of their duration. It has been proposed that memory formation can be divided into distinct phases, each phase involving specific neural changes at specific times after training. Research in this area involves interventive experiments in which various amnestic agents, which block specific neural processes, are administered to the experimental animal at specified times before and after the learning process, with subsequent analysis of the ability to show recall for the learned task.

The models that arise from these lines of investigation do not all agree in every respect, some are based on a two memory model system, while a three and more recently a four phase model system have been proposed. One of the earliest people to divide memory into phases was James (1950), who distinguished between short-term memory and long-term memory. He noted, that 'as a rule, sensations outlast for some time the objective stimulus which occasioned them'; this corresponds to the physiological after-image, and our consciousness of the after-sensation, which he terms 'elementary or primary' memory. If a stimulus lasts for a sufficient length of time it produces a more durable image, which may pass out of consciousness, but which can be recalled to it later; James termed this memory 'proper or secondary' memory. A comparable model in physiological terms had been given by Hebb (1949), whose two-stage theory of memory has gained widespread, though not universal, acceptance. Hebb postulated 'that incoming stimulation sets up a reverberatory activity (the activity trace) in the receptor and effector cells involved in the sensation; such

activity would be unstable and susceptible to interference from concomitant or later activity. However with repeated stimulation and reverberation from a specific percept, structural changes occur (the structural trace), perhaps in the form of synaptic or dendritic growths, which facilitate neural connections and therefore the firing of the sequence on a later occasion'. Hebb argued that the fundamental event was synaptic use associated with the firing of the post-synaptic neurones. The dichotomy of 'the active trace' and 'the structural trace' in describing memory is now more often referred to as 'short-term and long-term memory' respectively, based on the definition that the former is available for seconds or minutes after the exposure to the information, while the latter may be present for hours, weeks or years. Long-term memory will only occur if there is an adequate physiological trace formed in short-term memory, often referred to as the process of consolidation.

A duplex model of memory in humans, incorporating a short-term memory store and a long-term memory store, was first proposed in the 1960's (Atkinson & Shiffrin 1968, 1971). This model has been investigated by giving psychoactive substances to human volunteers. For example, Mewalt et al (1983) found that when diazepam is given to volunteers, short-term and retrieval from long-term memory remain intact, but the transfer of information from short-term to long-term memory is greatly impeded. According to the duplex model, new learning cannot be permanently encoded without the transfer or consolidation of information.

Frieder and Allweis (1982a,1982b) have proposed a four-phase model of memory formation, based on investigations which interfered with the neurochemical processes of memory formation in the rat. After training on an active avoidance task, rats were injected

intra-cisternally with various physiological inhibitors at specific pre- and post training times. These four phases have been classified as (1) very short-term memory, (2) short-term memory, (3) medium-term memory and (4) long-term memory, and are based on the differential effects specific physiological inhibitors have on the ability of the animal to show recall for a learned task at specific times after training.

The model of distinct phases of memory formation which has been extensively studied from the physiological aspect is the three phase model proposed by Gibbs and Ng (1977), who studied taste aversion learning in young chicks. Their work involved similar interventive research to that previously mentioned, and led to the classification of memory into (1) short-term memory, which lasts for minutes, (2) labile memory, lasting for up to thirty minutes and (3) long-term memory which may last for days. Subsequent to this classification, research proceeded to determine the physiological mechanisms underlying each phase and the times of their formation. It is proposed that increased electrical activation in neurones is involved in the development of short-term memory, followed by chemical activation in the cytoplasm; this includes an increase in Na^+-K^+ ATPase activity and increased intracellular calcium levels, which are the major processes in labile memory formation. Finally, long-term memory is thought to involve longer lasting changes, with an increase in protein and glycoprotein synthesis in the cell. Table 1.2 summarizes data on the time intervals, physiological mechanisms and inhibitors associated with each phase in the three phase model of memory.

Table 1.2

- The Three Stage Model of Memory Formation

Memory	Time	Inhibitor	Physiological Mechanism	Reference
Short-Term	10 mins	KCl/LiCl Electrosch.	Impulse Activity	Watts & Mark'70 Rose & Harding'83
Labile	30 mins	Ouabain	Na+/K+ ATPase Ca2+	DeVaus et al '80
Long-Term	Day/s	CX. Anisomysin	Protein Synthesis	Mark & Watts'71 Gibbs & Ng'77

Table 1.2. The three stage model of memory formation, showing time course and proposed physiological mechanisms. The specific inhibitor(s) used to block each stage is also given, together with associated references.

If we accept the three phase model of memory formation, or indeed any of the other proposed time-dependent models, a question arises; are the memory phases physiologically sequentially dependent, or do they occur in parallel?. By testing the animal's ability to show recall at various times after training, one can determine if the specific phases have has been formed, but this tells us little about the physiological mechanisms involved. Are the proposed mechanisms for each phase specific to its formation in a sequential manner, with initial neural activation leading to chemical changes, with a consequent increase in protein synthesis, in a time sequence similar to that observed for the formation of the different memory phases? Or are the physiological mechanisms occuring in a parallel manner; are the processes which are necessary for the establishment of labile and long-term memory occuring simultaneously with those involved in the formation of short-term memory? Figure 1.1 outlines the proposed relationship between the time of initiation of the physiological process in each phase of memory formation in (1) the sequential model and (2) the parallel model.

The relationship between the various stages are still very unclear, as the literature is scattered with evidence for both the sequential and parallel models. Among those opting for the sequential process of memory formation are Hebb (1949), Gold & McGaugh (1975) and Gibbs & Ng (1977). The plausibility of such a system gains support from several known features of learning. McGaugh (1966), who administered strychnine (a central nervous system stimulant), found that when given in subconvulsive doses learning in the rat was enhanced, which may indicate a mechanism of action associated with an increase in neuronal reverberation. A short-term memory of this type, persisting for hours, could then provide ample time for the establishment of major changes in synthetic systems, which could then

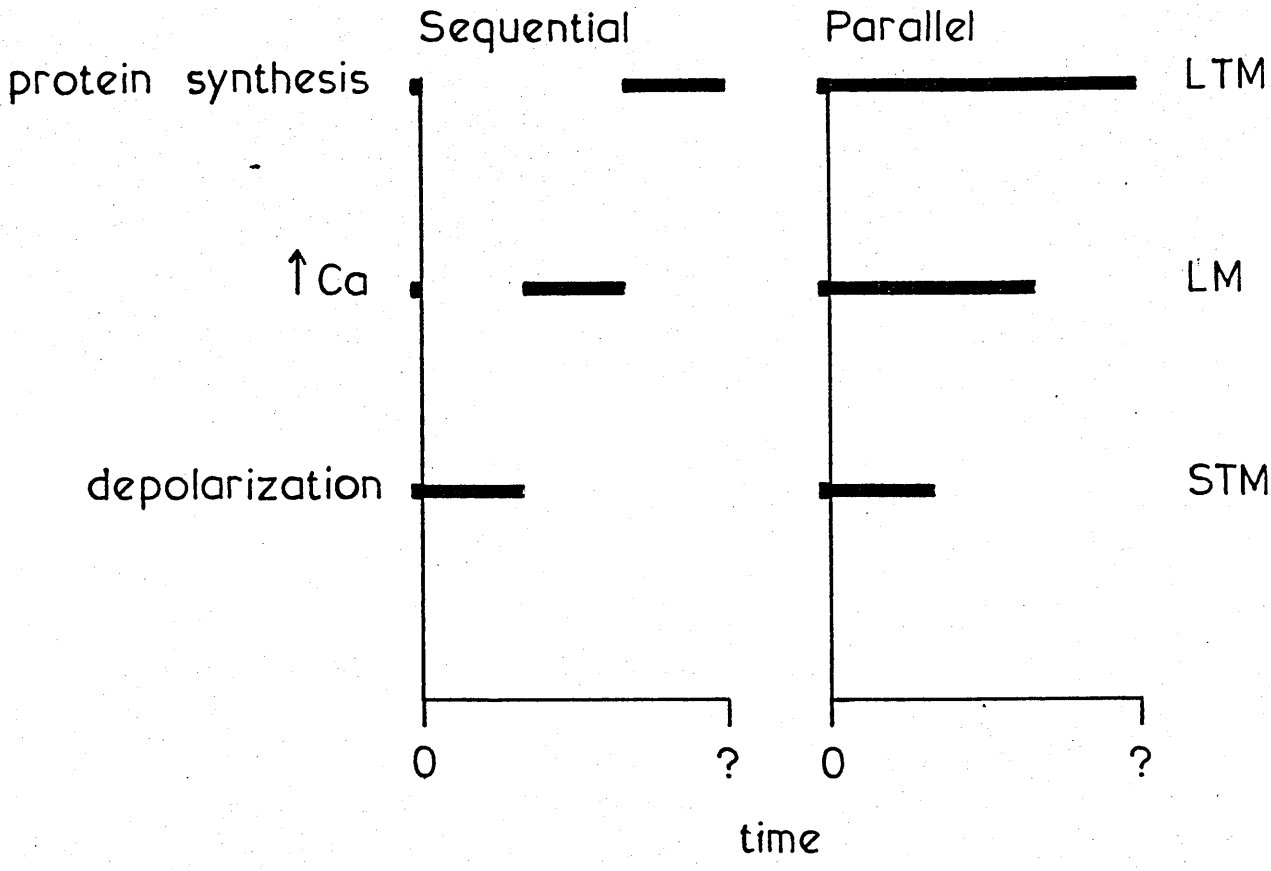


Figure 1.1. A diagrammatic summary of the sequential and parallel models of memory formation, with the physiological mechanisms postulated for each stage. In the sequential model, the physiological mechanisms occur in a sequence; labile memory (LM) and long-term memory (LTM) follow short-term memory (STM). In the parallel model, the mechanisms of LM and LTM occur together with those of STM.

constitute a long-lasting basis for the establishment of long-term memory. The flexibility of this model provides interpretation of the different effects of additional training on memory formation (Barondes & Cohen 1967). If training is minimal, then short-term memory dissipates before long-term memory can be established, and conversely, if training is prolonged, long-term memory is formed. In addition, Gibbs & Ng (1977) have shown that, if memory formation is a process of sequential phases, then disruption of any phase prior to its

initiation should prevent input of information into that and any succeeding phase, from any preceding ones, but should not have any effect on the preceding phases.

There is also evidence in favour of a model of the parallel phases of memory formation in the literature, but this evidence is less convincing, basically due to difficulty in determining at what time short-term memory is actually established. In this model, it is assumed that one set of physiological changes are mediated independently of another set, and that neither is necessary for the normal development of the other. If memory processes were arranged in parallel it might be possible to impair short-term memory but to allow long-term memory to develop normally. According to this model, only a relatively short period is required to allow sufficient synthesis for the establishment of a long-term memory trace. Observations which support this theory are available; for example, injections of acetoxycycloheximide (a protein synthesis inhibitor) given to mice 30 minutes after the end of training have no effect (Barondes & Cohen 1966), and Zemp et al (1966) found that RNA synthesis is accelerated during a learning experience and reverts back to normal fifteen minutes thereafter. Kessner & Conner (1972) have shown that post-trial electrical stimulation of the mid-brain reticular formation in rats disrupts short-term memory formation without affecting long-term memory, and conversely, that post-trial electrical stimulation of the hippocampus disrupts the long-term memory system without affecting short-term memory. These data strongly suggest that the structure for processing information is provided by a parallel activation of short-term memory and long -term memory. Reviews on this aspect of memory formation include those by Agranoff et al (1978), Roberts & Flexner (1969) and Matthies (1978); all favour a model in which a learning experience causes the formation of a

short-term memory and at the same time initiates new synthesis of macromolecules which will participate in long-term memory (if a long-term memory is to be formed).

However, neither of these two models can independently account for all of the experimental observations, and Kessner (1973) who once strongly supported the parallel processing hypothesis, was the first person to propose another theory. In order to account for the fact that disruption of short-term memory often produces disruption of long-term memory, he proposed that memory formation involves a combination of the two processes, a sequential process which is superimposed upon a parallel process. He suggested that information is initially transferred in parallel to the short-term and long-term memory systems, but that a sequential transfer of information is occurring at the same time.

1.5 A PROPOSED PHYSIOLOGICAL HYPOTHESIS

I wish to try and collate the evidence for a general, basic hypothesis which may shed some light on the possible mechanisms of neuronal modulation of connectivity , from a neurobiological perspective, that may occur as a result of a learning process and subsequent formation of a memory store.

Central and basic to studies on the cell biology of learning and memory is the assumption of increased neuronal activity consequent to a learning process. However, the exact location in the vertebrate brain in which this increased activation takes place is still largely unknown. Increased activation has been extensively studied using Aplysia, in which there is direct accessibility to large neurones, which are known specific sites of learning from which electrical potentials can be directly recorded. Increases in neural impulse activity are thought to be associated with the formation of short-term memory. The measurement of electrophysiological changes has also been investigated in isolated neuronal tissue in-vitro with particular reference to the rat hippocampus, (Lynch et al 1979; Voronin 1983).

Measurement of initial evoked responses shows that they trigger more prolonged reverberations of neuronal activity which can last for as long as several days. This is termed long-term potentiation (L.T.P.), and is elicited when high frequency stimulation of fibre systems in the mammalian hippocampus produce increases in the duration of the post-synaptic response activity. Indeed, there is mounting evidence that this neuronal activation may be the substrate for long-term memory. That L.T.P. is confined to synaptic complexes, is initiated by changes in intracellular calcium, and that a specific synaptic membrane protein shows endogenous phosphorylation upon high frequency stimulation, suggest that it may be a model for long-term synaptic

plasticity in memory formation (reviewed by Lynch & Baudry 1984).

Associated with neuronal activation are several chemical changes which occur at the synaptic site, including increased levels of intracellular calcium. In sensitization studies on Aplysia, Castellucci and Kandel (1976) have shown that there is enhanced transmitter release due to an elevation of cyclic adenosine monophosphate (C'AMP),with the activation of a protein kinase leading to the closure of potassium channels (see also Castellucci et al 1980, 1982). Closing these channels prolongs the action potential and thereby increases calcium influx (Klein & Kandel 1978, Klein et al 1980). Further to these studies, Alkon et al (1983), working with Hermissenda, propose that the mechanism of learning (sensitization, conditioning and habituation), at the cellular level, lies with a decrease in the K^+ current conductance via C'AMP and a calcium-calmodulin dependent protein phosphorylation, leading to a broadening of the action potential (reviewed by Hawkins 1984). The evidence that calcium is involved in the formation of L.T.P. is provided by Lynch et al (1983) for rat hippocampal preparations; the removal of calcium from the synaptic site with the specific chelator ethyl-diaminoglyco-tetra- acetic acid (EGTA) prevents L.T.P.formation.

Following these results, researchers have looked at the specific effects of increased intracellular calcium levels and it's modulation. Consequent to training chicks on a passive avoidance task, Rose & Tillson (unpublished results) have found an increase in calmodulin (a calcium regulatory protein) in brains from trained chicks compared to controls. Calmodulin is thought to play a pivotal role in intra-cellular calcium regulation (Cheung 1980). Further studies by Lin and Way (1984) have characterised specific calcium activated ATPases which are located in brain nerve endings,and Hoch and Wilson

(1984), investigating phosphorylation of proteins in the hippocampus in-vitro, have shown that calcium may have a specific role to play. Changes in the chemical composition and activation of protein kinases are thought to be associated with formation of labile memory.

Popov et al (1976a) found that, as a result of training rats, there is an increase in fucose incorporation into brain glycoproteins, and concomitant with this, an increase in fucokinase activity (this enzyme is involved in the synthesis of fuco-glycoproteins: Popov et al 1983). Similar results have been obtained by Burgoyne and Rose (1980a), who found an increase in fucose incorporation in particulate proteins in chicks trained on a passive avoidance paradigm, with an increase in fucokinase activity (Lossner and Rose 1983).

The calcium dependence of fucokinase activity in the chick is addressed in Chapter 3 of this thesis.

This increase in fucose incorporation may indicate that; (1) there is an increase in fucosylation of pre-existing proteins, or (2) an increase in fucosylation of proteins synthesised de-novo. I have tried to resolve this distinction by experiments reported in Chapter 5 of this thesis.

Coupled with changes in glycoprotein metabolism, there are numerous studies which indicate an increase in protein synthesis as a result of a learning process (Smith and Yarwood 1970; Schliebs et al 1985; Hambley et al 1977). Increases in receptor number have also been found (Longstaff and Rose 1981; Lynch et al 1982), and it is thought that these changes which are observed as early as thirty minutes after training and persist for up to twenty-four hours, may be associated with the formation of long-term memory. Evidence that memory formation may be ultimately concerned with such post-translational modification of proteins (that is, any modification

of the protein following release from the ribosome), has been obtained by many investigators (reviewed by Routtenberg 1982). Included in such modification is the process of phosphorylation of membrane proteins; in particular, Routtenberg & Lovinger (1985) have found a selective increase in phosphorylation of a 47 kDa protein (F1) which may be directly related to L.T.P. effects.

That such changes are found in membrane fractions suggests that they may be associated with neuronal remodelling and/or changes in neuronal connectivity. Evidence of structural changes has been found in morphometric studies of tissue from trained animals; in the chick, Stewart et al (1984) have found increases in pre-synaptic volume density in specific areas in the brains of trained chicks, and Bradley et al (1981) have found a change in the length of the synaptic apposition zone in the left hemisphere of chicks after an imprinting experience.

Do these neuronal morphological changes indicate evidence of neuronal plasticity and result from specific physiological changes associated with learning and memory formation?

From all the research into the the cell biology of learning and memory, using a specific experimental design, one can tentatively draw conclusions to link the various results into a plausible, working theory. Many recent reviews do exactly this (Dunn 1980; Lynch and Baudry 1984; Thompson et al 1983; Booth 1973; Rose 1981; Rosenzweig 1984). Although a review of the literature is very constructive, I think one must be aware of the danger of oversimplification and misguided extrapolation; an hypothesis must be regarded as tentative and be subject to verification and modification.

The main reason for this skepticism is the realization that in approaching a study on the biochemical correlates of memory formation, one is primarily looking for biochemical processes specific to learning, over those physiological and developmental changes which are already present. The inability to separate these two cell-biological effects is often very difficult.

This thesis is concerned with investigating further the biochemical correlates of passive avoidance training in the chick. It is a continuation of the work of Rose and colleagues at The Open University. I have been primarily concerned with the post-translational modification of proteins, in particular fucose containing glycoproteins, consequent to training.

CHAPTER 2

LEARNING AND MEMORY IN THE CHICK

Since the pioneering work of Cherkin (1969) and Watts & Mark (1971a, 1971b) , the young chick has proved to be a useful model for investigating neurobiological processes underlying learning and memory formation in a variety of tasks, including imprinting and passive avoidance learning. However, before embarking on an investigation of the cell biological implications of any behaviour, it is first necessary to consider the structure and function or the avian brain.

2.1 STRUCTURE AND FUNCTION OF THE AVIAN TELENCEPHALON.

Beginning with the initial research of Flourens (1824), numerous investigators have used brain lesions to explore the functional significance of various regions in the avian central nervous system (reviewed by Pearson 1972). It is largely from this interventive research that attention has been focused on the avian cerebral hemispheres; the telencephalon (as in mammals) appears to be the critical area involved in the organization of complex behavioural patterns including learning and memory (Rogers 1922). However, these initial studies were inadequate as there were no standard anatomical descriptions available of the basic structure of the telencephalon, so lesions could not be localised. Approximately twenty years ago with

the development of modern neuroanatomical tracing techniques, clearer pictures of the general organization of neuronal pathways were made available with the publication of the avian brain atlases of Karten & Hodos (1967) and Zeir & Karten (1971). Armed with this critical information and the attendant insights gained from brain lesions and electrophysiological studies, a better understanding of the development and involvement of the telencephalon in mediating complex behaviour was gained.

In contrast to the laminar organization of the mammalian cortical mantle, the avian telencephalon is characterized by broad fields of cells, some nuclear clusters and a thin, overlying laminated zone. Until the late 60's, most comparative anatomists believed that the bulk of the telencephalon in birds and reptiles, was equivalent to the mammalian basal ganglia (Kappers et al 1963). The increased tissue mass of the telencephalon of birds and modern reptiles compared to that of amphibians and ancestral reptiles, was thought to reflect an expansion of nuclei equivalent to those of the mammalian ganglia. In contrast, the increased tissue mass of the mammalian telencephalon as compared to that of amphibians and ancestral reptiles, was thought to reflect the proliferation of a new type of telencephalic tissue, the neocortex. This prevailing theory of comparative telencephalic organization was not widely challenged until recent experimental studies began to demonstrate that only a restricted portion of the avian telencephalon, the ventrolateral wall possessed morphological and histochemical characteristics similar to those found in the mammalian basal ganglia (Karten & Dubbledam 1973). From the evidence it seems that the avian and mammalian telencephalon share a common ancestry with respect to some of the major features of telencephalic organization.

Both the mammalian and avian telencephalon develop as a set of lateral evaginations from the rostral end of the neural tube. Each evagination closes back upon itself forming the tubular processes of the two cerebral hemispheres. The five major constituents of the avian telencephalon are structures which are labelled as 'striata': the paleostriatum, the archistriatum, the neostriatum , the ectostriatum and the hyperstriatum. Figure 2.1 shows cross-sections of the pigeon telencephalon, indicating the location of these and other structures.

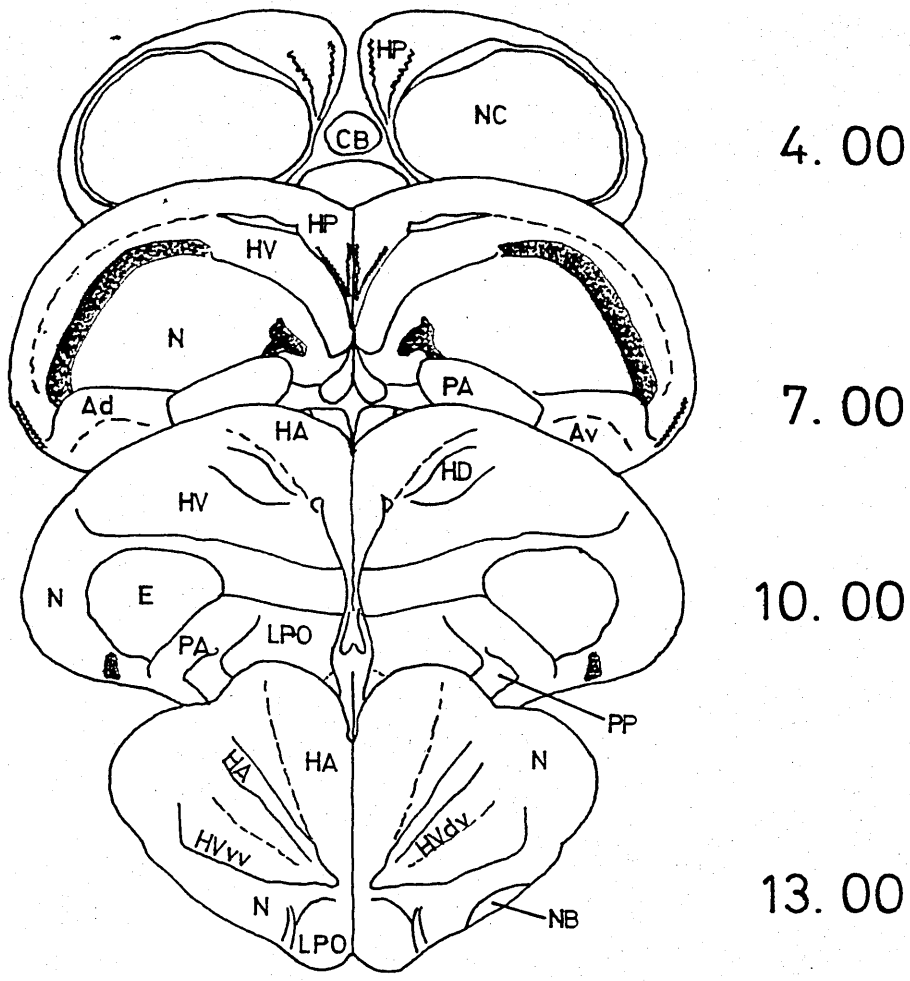


Figure 2.1. Transverse sections through the telencephalon of the pigeon; the figures to the right of each section indicate location (measured in millimetres anterior to the interaural zero, using the vertical plane of the atlas of Karten and Hodos, 1967). See Abbreviations List for further information.

Although it was initially believed that at least the first four of these regions were homologous with parts of the mammalian basal ganglion, more recent embryological studies (Kallen 1962) have disputed this. These indicate that only the paleostriatum derives from the basal part of the embryonic telencephalon, and all the other avian 'striatal' structures derive from the dorsal part and so are in fact of pallial origin. As is evident from Figure 2.1, the largest single cell mass in the forebrain is the neostriatum, which is divided into three regions; the neostriatum frontale, intermedium and caudale. There is evidence that the caudal neostriatum is involved in hearing (Karten 1968), but little is known of the functions of the other two anterior parts. The ectostriatum lies laterally and ventrally in the anterior neostriatum, separated from underlying structures by a fibrous layer, the dorsal medullary lamina. This region supports a direct projection from the thalamic nucleus rotundus. Overlying the neostriatum, and separated from it by the lamina hyperstriatica, is the hyperstriatum ventrale, which like the neostriatum, appears to possess primarily (but not exclusively) intrinsic connections to and from other telencephalic regions. It lies midway between the anterior and posterior forebrain and extends approximately 2.4 mm anteroposteriorly. Structurally, it is subdivided and separated by the lamina frontalis superior from the hyperstriatum dorsale and the hyperstriatum accessorium. The latter two regions, which are divided by the lamina frontalis suprema, form the so-called 'wulst', an elevation of the hemispheres, and are thought to be associated with vision. However, the function of the hyperstriatum, incorporating ventrale, dorsale and accessorium, is currently the subject of active speculation and experimentation, as there is increasing evidence implicating it in the processing of complex behaviour. Experiments involving hyperstriatal lesions in the

chick (Rogers 1922; Benowitz 1972) and in the pigeon (Zeigler 1963) have shown deficits in reversal learning, simple discriminations and position learning. In correlative experiments, many physiological changes have been found in this area as a result of a training procedure.

The archistriatum occupies a ventro-lateral position in the caudal third of the hemisphere below the neostriatum. This is a very complicated region structurally, with four major subdivisions each containing four to eight discrete nuclei. This area is thought to be associated with agonistic responses, stimulation of the medial archistriatum inducing escape behaviour in mallards; destroying this area drastically reduces 'fearfulness' (Phillips 1964). Similar effects have been noted by Cohen (1975) in pigeons and Benowitz (1972) has reported that chicks with archistriatum lesions are unable to refrain from pecking at an aversive stimulus. (In the classification of birds, some 8,600 species have been arranged into 28 orders (Storer 1971). Avian taxonomists conveniently list these orders in such a way as to reflect a ranking of birds, from least evolved and so most primitive to most evolved. The mallard, of the order Anseriformes, the chick (Galliformes) and the pigeon (Columbiformes) arranged in ascending order of evolution, are sufficiently similar phylogenetically, to allow real comparisons to be made about anatomical function).

The principal subpallial structures are the antereolateral nucleus basalis, the lobus parolofactorius (which merge with the paleostriatal complex) and the medially located septal area. The lobus parolofactorius receives secondary olfactory fibres from the olfactory bulb and may be comparable to the mammalian limbic system (Karten & Dubbledam 1973). Like the caudate-putamen, the

paleostriatal complex and the lobus parolofactorius contain mostly small neurones which have high levels of acetyl cholinesterase and dopamine (Bertler et al 1964). The adjoining paleostriatum complex contains three structures, the outermost being the paleostriatum augmentatum which envelops the paleostriatum primitivum, within which lies the nucleus intra-peduncularis. There is evidence indicating that the augmentatum is homologous with the mammalian caudate-putamen complex (Karten & Dubbledam 1973), in that both are rich in cholinesterase and dopamine and receive major afferents from overlying pallial regions. In a more recent review by Reiner et al (1984), on the distribution of enkephalin within the mammalian basal ganglia and the avian paleostriatal complex, results confirmed that the paleostriatum primitivum is comparable to the mammalian globus pallidus and that the paleostriatum augmentatum and the lobus parolofactorius are comparable to the caudate-putamen. As will be discussed later, recent behavioural experiments have indicated that the lobus parolofactorius and some regions of the paleostriatal complex may be involved in learning and memory processes.

Although there has been immense progress in the development of accurate avian brain atlases, the only two areas which have been extensively studied and functions outlined are the archistriatum and the hyperstriatal complex, using specific lesioning and biochemical methods. Damage to the latter complex disrupts a variety of types of learning, but the precise nature of these deficits is not clear. (The biochemical changes found in this region as a result of learning and memory will be discussed later). There is good evidence that the archistriatum is (as in reptiles and mammals) of critical importance in fear- motivated behaviour, but little understanding of other functions of this structure is available. There is scattered evidence of the involvement of other brain areas in conjunction with the two

previously mentioned in complex behavioural processes, but in general, more intensive investigations are needed in this area.

2.2 LATERALIZATION OF FUNCTION

It is becoming increasingly evident that lateralization of brain function, once thought to be unique to humans, is present also in the avian brain. Functional asymmetry has been demonstrated clearly in the avian brain, both by monocular testing (Andrews et al 1981), and by unilateral administration of either cycloheximide (Rogers et al 1974) or glutamate (Howard et al 1980) into either forebrain hemisphere on day 2 of post-hatch life. It has been observed that after a single dose of either of these substances, to the left hemisphere, retarded visual and auditory learning is evident. Similar treatment of the right hemisphere is without effect (Rogers & Anson 1979). These workers have suggested an ontogenetic mechanism which may account for this lateralization observed in chicks, which may be associated with unilateral sensory input during development of the forebrain hemispheres. They propose that light experience may have an important role to play, in that after day 17 of incubation the embryo is orientated in the egg such that the left eye is occluded by the wing and body. The right eye, however, is next to the air sac and exposed to light input (Oppenheim 1978). As the optic nerves decussate completely most of the information passing through each eye is processed by the contralateral hemisphere. It is possible that light entering the right hemisphere stimulates the developmental processes in the left hemisphere in advance of the right. When chicks were hatched from eggs incubated in darkness, no functional asymmetry in the forebrain was found (Rogers 1982). Probably the clearest example of lateralization in the avian brain is seen in some species of passerine birds. In the adult male chaffinch, white-crowned sparrow and canary, transsection of the left hypoglossal nerve causes loss of almost all song components, whereas right transsection has little effect (Nottebohm & Nottebohm 1976).

Not only is there evidence of lateralization, but there are reports which suggest that the degree of hemispheric lateralization in males and females is different. In a series of experiments analyzing response to visual stimuli, using the rate of bead pecking with right and then left eye occluded, a measure of habituation was recorded. Andrews et al (1982) showed that there were marked differences between the eye systems. In females, the left eye system (LES) and the right eye system (RES) were similar in showing habituation, while there were marked differences in LES over the RES of males. The LES showed greater specialization and is thought to be associated with the function of spatial analysis (reviewed by Andrews 1983). Thus, as in humans, there seems to be differential specialization in the two hemispheres of the avian brain, and increasingly there are reports of lateralization of change, both biochemical and morphological, in the chick after training. This lateralization of function has also been investigated in experiments reported in this thesis.

2.3 THE CHICK AS AN ANIMAL MODEL FOR NEUROBEHAVIOURAL RESEARCH.

The domestic chick has been used extensively as an animal model for research on the biological basis of behaviour, as it possesses many unique characteristics. The young chick has a versatile response repertoire which includes initial stimulus preferences, imprinting and the ability to learn quickly in many conditioning situations. Most importantly, the chick is able to feed itself immediately after hatching and consequently many problems regarding maternal influences and separation distress are attenuated. A main advantage of using this animal as a model in psychological research is that the chick is more mature at hatching than most mammalian species are at birth; for example, behavioural changes occur much more rapidly in the precocial chick than in the altricial rat. Significant behavioural changes in the chick occur within the first three to four days after hatching, whereas in the rat comparable changes are evident as late as the second and fourth week after birth (Zolman et al 1976). From a behavioural point of view this is very advantageous, as chicks can be hatched and reared in darkness until they are exposed to a particular learning paradigm or stimulus; although it would be naive to suppose that the nervous system is relatively inactive, the chances of detecting changes that are specific to a learning task are maximized. The main draw-back with working with a young animal is that essential neuronal developmental processes will inevitably be occurring, which could lead to serious problems in interpretation of data from behavioural experiments.

Other characteristics of chick physiology which make these birds useful in psychopharmacological research include; (1) the blood brain barrier is not fully developed, allowing penetration to the brain of substances which are excluded in adult animals (Purdy & Bondy 1976)

and (2) the enzymes which inactivate various transmitters are present in low concentrations in young chicks, so that the central effects of such transmitters may be investigated (Marley & Stephenson 1972).

In this chapter I wish to discuss two specific learning paradigms which have been extensively studied in the chick: imprinting and passive avoidance learning (with special emphasis on the latter, as it is the training task on which the work in this thesis was based).

2.4 IMPRINTING

Various groups have worked with the young chick exploiting imprinting behaviour and visual experience (reviewed by Bateson 1976). The term 'imprinting' was first used by Lorenz (1935), who observed that geese bred in an incubator follow the first moving object they see after hatching. The object need not be a conspecific; it can even be a person handling the bird. This preference for the first moving object proved to be stable for the entire period during which the following behaviour persisted (filial imprinting). The time in which the preference is formed is called the 'sensitive period'. The conclusions Lorenz formulated were the basis of subsequent research designs on the processes of imprinting which have confirmed and disproved parts of his theory.

Imprinting, which is often regarded as a special type of learning, clearly represents a very important experience for the young bird which influences subsequent behaviour. It is a very convenient training procedure to use, as many aspects can be controlled and manipulated, so allowing more accurate measurement of neurobiological changes associated specifically with imprinting.

Bateson, Horn and their colleagues in Cambridge, and Rose at The Open University have devoted many years of research to the neural changes consequent to imprinting in the young chick, and have produced many elegant results. The methods used in the early experiments for hatching, rearing and training the chicks were constant, with only minor modifications in later experiments. Eggs were incubated and hatched in darkness in individual houses until they were sixteen hours old. They were divided into three groups; one group remained in darkness and served as 'dark controls', another group was exposed to overhead illumination ('light controls') and the third group, '

experimentals', was exposed to a flashing orange light. Later experimental designs involved varying times of exposure to the flashing light; either forty-five minutes (undertrained chicks) or one hundred and eighty minutes (overtrained chicks), to reduce certain non-specific factors including differences in sensory stimulation. Further modifications in the later experiments in which preference studies were carried out, involved two different imprinting stimuli, a flashing red light and a stuffed jungle fowl. The major objectives of the biochemical studies were to answer the following specific questions: (1) is there a biochemical change in the brain associated with imprinting? (2) is the change a side-effect of the training procedure, or is it directly related to the imprinting process? (3) is the change localized to a particular part of the brain?.

The first experiments (Bateson et al 1969, 1972) were essentially exploratory, attempting to locate specific forebrain regions in which biochemical changes occurred. These initial experiments involved dissection of the brain into three areas; the dorsal forebrain roof containing major visual projection areas (Karten et al 1973), the forebrain base, which contains structures concerned with the control of motor function (Zeir & Karten 1971) and the midbrain, which includes the optic tectum. Figure 2.2 indicates the positions of these dissections.

Early experiments analysing the rate of incorporation of labelled uracil and lysine into specific brain areas showed that seventy-six minutes after training there was an increase in incorporation into RNA of the experimental chicks over controls. This increase was localized to the forebrain roof (Bateson et al 1972). Subsequent follow-up experiments to reduce the possibility that the observed changes were due to non-specific effects were performed by Horn et al (1973a), and previous results were confirmed. In order to determine the

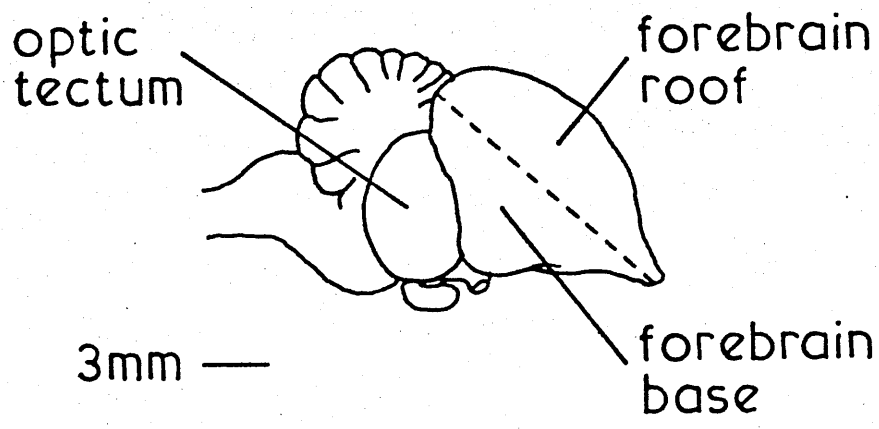


Figure 2.2. Lateral view of the chick brain. The regions used in studies of fucose incorporation were (a) the dorsal part ('roof') of the forebrain, separated from (b) the ventral part ('base') by a cut along the plane indicated by the broken line, and (c) the midbrain, which includes the optic tectum (redrawn from Horn, 1981).

localization of biochemical change consequent to imprinting, Horn & McCabe (1979) conducted an autoradiographic study to detect areas of labelled uracil incorporation. Significant differences in incorporation between experimental and control chicks were found in only one region, the intermediate and medial part of the hyperstriatum ventrale (IMHV), located in the forebrain roof. Evidence of a similar localization of increased metabolic activity in chicks trained on the imprinting paradigm was obtained by Kohsaka et al (1979) using the 2-deoxyglucose method (in which labelled 2-deoxyglucose is injected into the animal and the region(s) of accumulation of the label or its

phosphorylated compound, 2-deoxyglucose-6-phosphate, are analysed). The finding of a localized change led to numerous detailed investigations of this area. Lesioning studies (McCabe et al 1981) and electrical stimulation studies of this area (McCabe et al 1979) showed that the integrity of the IMHV was essential for imprinting to occur. Morphological studies by Bradley & Horn (1978,1979) and Bradley et al (1981) showed that there was a hemispheric asymmetry in the IMHV when the chicks were exposed to an imprinting stimulus. In 'undertrained' chicks, there was a hemispheric asymmetry in the length of the synaptic apposition zone, the right having greater length than the left. This asymmetry was eliminated by further training, as seen in 'overtrained chicks', where there was no difference in synaptic apposition length between hemispheres. This morphological change may indicate a strengthening of synaptic coupling as a result of imprinting. From this elegant research (which is still in progress), a model of engram formation and location has been proposed. It is thought that the left IMHV is probably the initial site of storage 'S' and that later the right IMHV becomes involved in the consolidation of the store 'S'' (Horn 1981). This lateralization of function to the left hemisphere has been documented previously by other workers and will be discussed in relation to experimental results reported in this thesis.

2.5 PASSIVE AVOIDANCE LEARNING

As imprinting is often regarded as a special type of learning in that it is ontogenetic, occurs during 'a sensitive period' and is irreversible, other researchers have studied an alternative learning task with the chick, a one trial passive avoidance learning paradigm (P.A.L.). The main advantages of this paradigm over imprinting are the speed of acquisition and the relative simplicity of training; consequently, one can investigate with greater (relative) accuracy the neurochemical correlates of memory, and in particular those processes associated with short-term memory.

This paradigm, introduced by Cherkin(1969) and subsequently modified by Gibbs & Ng(1977), has been used extensively by the latter group and is the paradigm on which the concept of the three stages of memory formation was based (discussed in Chapter 1). It has also been used in my laboratory and is the paradigm on which this thesis is based.

The passive avoidance learning paradigm is a task in which the animal is trained to avoid a distasteful stimulus by not performing a particular response: hence the term 'passive'. Ross Chunky chicks are hatched in a communal brooder with a twelve hour light/ dark cycle and at a constant temperature of 37 C. The chicks are marked on hatching so that accurate age records can be kept. Chicks are then sexed by the method of Serebrovsky (1966), which exploits the fact that at a very early age, differences in wing-feather lengths are obvious in one sex. Female chicks have typically uneven wing-feathers while the male's are completely uniform. It is interesting that there is also sexual dimorphism in the timing of hatching ; females tend to hatch earlier than males (Davies & Payne 1982). The importance of sexing the chicks has only recently been acknowledged, as evidence is

available which indicates that there may be marked differences in hemispheric development between males and females. Also, van Haaren & van de Poll (1984) have noted that there is a difference in the performance of male and female rats trained on a passive avoidance task, which may be attributed to differences in levels of testosterone at the time of training. Female rats were shown to have a lower success rate in training compared to males, but the number of confounding variables are so numerous that it is difficult to draw any conclusions at this stage; this area of behavioural endocrinological research is still in its infant stage.

When the chicks are 18 - 30 hours old they are transferred, in pairs, to aluminium pens (measuring 26 x 25 x 20 cm), each illuminated by a red bulb and at a temperature of 28 C. The chicks are paired to reduce any stress which may arise due to isolation and one of each pair is marked for identification purposes. Each pen is sprinkled with food pellets and the chicks are left to habituate to the pens for one hour before training, after which time they show normal exploratory and locomotor behaviour. This period of time is essential, as the chicks are obviously initially stressed and very noisy which could influence subsequent behaviour.

Training is carried out at the same time each day, begining at 08.00 hours, in order to avoid any changes in behaviour due to diurnal rhythmicity. Radford & Armstrong (1981) observed that learning and memory for a single trial passive avoidance behaviour in day-old chicks may be influenced by the time of day at which training takes place. They concluded that there was a complex diurnal rythmicity in arousal and pecking activity which affect learning performance and memory.

Training begins with two presentations of a white bead (measuring 3 mm in diameter), suspended on a long rod, with an interval of five minutes. Before presentation of the bead to each chick, which lasts for ten seconds, the sides of the pens are tapped lightly to focus attention on the training stimulus. After each presentation of a bead the behaviour of each chick is scored on a 'peck' or 'no peck' basis. Five minutes later the chicks are presented with a chrome bead (measuring 4 mm in diameter), and again behaviour is noted. These three trials are the pretraining trials, the purpose of which are to familarize the chicks to pecking presented beads. Ten minutes later the training trial begins; two chrome beads (similar to those used in the pretraining trial) are used, one coated with water (W) and the other coated with methylanthranilate (M), which is distasteful to the chicks. Both birds in a single pen are presented with either a water coated bead or a methylanthranilate coated bead. Again, each presentation lasts ten seconds and the behaviour of each chick recorded. Characteristically, those chicks trained on the (W) bead showed an avid peck response while those chicks trained on the (M) bead initially peck and then exhibit a 'disgust response', involving vigorous head-shaking. At the chosen test- trial interval the chicks are tested for behaviour on presentation of a dry chrome bead. Those chicks which were trained on the water coated bead and which peck on the test trial are considered as 'controls' or water (W) controls, while those trained on the methylanthranilate coated bead and which avoid the bead on the test trial are taken as the 'experimentals' or methylanthranilate (M) trained. It is assumed that the experimental chicks have learned that the bead is aversive and will subsequently not peck it, thus exhibiting passive avoidance behaviour. Within any training session, the proportions of chicks which show behaviour fulfilling 'control' and 'experimental' criteria are 75% - 80% and 70%

- 80% respectively.

This training regime is convenient and relatively short but as with any behavioural test, it has numerous draw-backs due to the presence of confounding variables which one simply cannot control. Indeed, as a neurobiologist, one must of necessity take a very reductionist view of behaviour, picking only one aspect and concentrating on any concomitant physiological changes. I have made reference to variability intrinsic in each animal's behaviour during training which may have confounding effects on behavioural biochemical studies in Chapter 1, and wish to expand on it at this point, with reference to the passive avoidance paradigm.

The problem of behavioural variability when using chicks as subjects has been recognised by Gottlieb (1971) and Gebotys et al (1983) with respect to their own work on imprinting, in which they found that there were separate subpopulations within a single hatch. Gebotys et al (1983), in an investigation on the following behaviour of chicks from the same hatch, found that the chicks could be divided into two subpopulations, and between hatches variability may arise as a consequence of genetic-developmental differences (Bateson 1974). Other factors which may contribute to variability are temperature and atmospheric pressure variations (seasonal variations), which may also cause differences in behaviour between hatches. I have also recognised that these factors exist and may affect the general locomotor activity and arousal of chicks, consequently influencing approach behaviour to the training beads. On pretraining, the time of presentation of the bead to each chick is standard (ten seconds), but the actual number of pecks by each chick is very different and cannot be controlled, leading to differences in locomotor activity. In some experiments, where the accent is on documenting specific behavioural

differences, record is noted of number of pecks by each chick, but I have had to overlook this aspect in my experiments. Another source of variability is the degree of distaste of methylanthranilate and the degree of thirst shown by the (M) trained and (W) controls animals respectively, which again will influence the number of pecks each chick makes. In the (M) trained group, chicks show varying degrees of stress and headshake, while differences in avidity in pecking the water coated bead are evident in the (W) control group. Another major factor is the behaviour of the partner (social facilitation), which can either encourage or discourage subsequent pecking. This is very obvious in (M) trained chicks, when a strong disgust reaction by one of the pair sometimes leads to immediate avoidance of the bead by the other chick.

However, we must of necessity ignore these variables and chicks which meet the criteria as outlined above are labelled (M) trained and(W) controls and are subsequently used for biochemical analysis. Many researchers have recognised the problems inherent in behavioural-biochemical studies and have tried to eliminate them by using large sample sizes and various standardization procedures before statistically analysing results. However, I think it is important to be aware that such intrinsic variability does exist in the behaviour of any group of animals, and that it may play a major role in the degree of reproducibility of behavioural-biochemical experiments.

2.6 THE CELL BIOLOGY OF PASSIVE AVOIDANCE LEARNING.

Arising from collaborative work on the physiological changes associated with imprinting, Rose et al (1980) embarked on research into the biochemical consequences of passive avoidance learning in the chick, to determine if similar biochemical sequences and anatomical areas are involved in a different type of learning behaviour.

Initial experiments examined (^{3}H)-quinuclidinyl benzilate (QNB) binding, (a measure of muscarinic-cholinergic receptors) in the chick forebrain and were stimulated by the observation that the chick brain is particularly rich in enzymes of the acetylcholine system. The forebrain was cited as an area critical for biochemical changes associated with imprinting (Bateson et al 1972). Thirty minutes after training there was a 21% increase in QNB binding to the forebrain membrane protein of (M) trained chicks over (W) controls. This increase was not observed ten minutes after training and had disappeared after three hours. This transient increase in muscarinic receptors suggested an early involvement of cholinergic synapses in the initial stages of learning and memory formation. These studies were followed by experiments which showed that there was an increase in nicotinic cholinergic receptor binding (Aleksidze et al 1981) as a result of training chicks on P.A.L.. Further experiments to determine if there was any change in protein metabolism, involved analyzing the incorporation of labelled L-leucine into particulate proteins in vivo. There was an increased incorporation into both the soluble and particulate protein fractions in the anterior forebrain roof of experimental animals. The increase was localized to a tubulin-enriched fraction (Mileusnic et al 1980). More recent experiments by Schliebs & Rose (1985) have shown that this increase can be replicated in vitro. One hour after the chicks were trained,

they were killed and forebrain slices (0.4mm thick) were incubated in a glucose containing medium containing ^{14}C-L-leucine at 37 C. An increase of 23% in leucine incorporation was found in the soluble fraction of tissue from (M) trained over (W) controls chicks.

Subsequent experiments concentrated on the effects of training on glycoprotein metabolism, in which fucose was used as a marker. Following intraperitoneal or intracranial injections of labelled fucose, chicks were trained, killed at various times after training and injection, and fucose incorporation analyzed. At 0.5, 3 and 24 hours after training, a significant increase in incorporation was observed in the particulate fraction of the anterior forebrain roof in trained chicks (Sukumar et al 1980). This increase was further localised to a synaptic membrane and mitochondrial fraction (Burgoyne & Rose 1980a). These experiments strongly indicated that there were some changes in protein and glycoprotein metabolism as a result of training, located in the forebrain roof. In order to ascertain if the observed changes in fucose incorporation were a direct result of training and not due to any other concomitant factor, (including increased stress due to the taste of methylanthranilate), Rose & Harding (1984) performed a very elegant study. This was based on the hypothesis that if chicks were trained and made amnestic, then there should be no observed changes in fucose incorporation if this effect is directly correlated with learning. From this series of experiments it was argued that increased fucose incorporation was indeed specific to memory formation, as no such change was noted in chicks that were trained and rendered amnestic by electroshock. Further experiments by Kossut & Rose (1984) have analysed the specific metabolically active sites in the chick forebrain associated with training, using the 2-deoxyglucose autoradiographic technique. One minute prior to training chicks were injected with the isotope and killed thirty

minutes after the test trial. Autoradiograms of coronal and parasaggital brain sections were scanned densitometrically and the grain densities of labelled structures measured. Regions which showed enhanced labelling in the (M) trained chicks over the (W) controls were the hyperstriatum ventrale, located in the anterior forebrain roof, the paleostriatum augmentatum and the lobus parolofactorius in the forebrain base. A more recent study by Rose & Csillag (1985) using ^{14}C-2-deoxyglucose has confirmed these findings. To distinguish effects of training from those of consolidation, and to study lateralization of the increased uptake of ^{14}C-2-deoxyglucose (2-DG), the label was injected intraperitoneally either five minutes before, or ten or thirty minutes after training. After thirty minutes bilateral samples of the medial hyperstriatum ventrale (MHV), lobus parolofactorius (LPO) and the paleostriatum augmentatum (PA) enriched regions were dissected. They found that there was an increased accumulation of 2-DG in the left LPO and in the left MHV of the (M) trained birds over the (W) trained, when the injection was given five minutes before training. With an injection ten minutes after training, there was an increase in incorporation in the left LPO, left MHV and the right MHV in the (M) trained chicks. Finally, when the injection of 2-DG was delayed until 30 minutes after training, the increase in the left MHV still persisted. These results indicate that the MHV and the LPO are important areas in which biochemical changes are found as a result of training chicks on the passive avoidance learning paradigm.

With all of this data on biochemical changes in the forebrain roof and base associated with training, attention was turned to stereological studies to determine if any morphological changes could be detected in specific regions: the MHV, PA and LPO. These experiments were designed to investigate any lateralized changes which

may exist before and after training. Twenty-four hours after training, chicks were anaesthetized and transcardially perfused with fixative. Brains were then prepared for sectioning. Specific regions from the right and left hemispheres were stereotaxically located with the aid of co-ordinates derived from the chick brain atlas of Youngren & Phillips (1978). From these studies, Stewart et al (1983) found that there were no significant differences in either hemisphere of (M) trained chicks over (W) controls in the numerical density of synapses (Nv) and the mean length of the post-synaptic thickening (D) in the P.A. and in the LPO. However there was a hemispheric difference in (D) in the right LPO over the left in (W) control chicks which was not evident in the (M) trained chicks. This rather puzzling result, of a decrease in the post-synaptic thickening in the LPO following training, was thought to reflect alterations in synaptic contact zones involved in transmitter release and binding. A subsequent study to investigate morphological changes in the medial hyperstriatum ventrale (MHV) after training was undertaken by Stewart et al (1984). Using the same technique, a number of parameters were measured; the number of synapses per unit volume of neuropil (Vv syn), the volume density of presynaptic boutons (Nv ves), the number of synaptic vesicles per unit volume of neuropil (ves.syn) and the mean number of synaptic vesicles per pre-synaptic bouton. Changes similar to those found in the LPO were found in the MHV; the post synaptic thickening (D) in the right MHV in control chicks was greater by 12% than that in the left, and this difference disappeared on training. This finding agrees well with that of Bradley et al (1981), who found that an asymmetric difference in the length of the synaptic apposition zone, the right being longer than the left, was eliminated by further training. Training also influenced the value of Nv.ves; in(W) control chicks, Nv.ves was 12% greater in the right than in the left

MHV, but following training these differences were reversed. Also following training, there was a 22% increase in the value of Vv.syn. in the left M.H.V. over the right after training in the (M) trained chicks. The most striking result, however, was that recorded for the number of vesicles per synapse (ves.syn.), which showed that, after training, there was a 61.4% increase in this value in the left hemisphere over the right in (M) trained chicks.

Interpretation of these data is rather complex and involves the consideration of asymmetric developmental differences in the naive chick and those associated with training. The increases observed in the Nv.ves. and the greater increase in the ves.syn. in the left hemisphere of the (M) trained chicks are thought to reflect changes in pre-synaptic terminal activity, including membrane-vesicle recycling and chemical transmitter release. Also, the question as to whether the biochemical changes previously documented as a result of training are associated with the observed morphological changes has still to be clarified. However, it is interesting to note that the lateralization of change is consistent with other reports that memory formation is localized initially in the left hemisphere and that later the right becomes involved (Horn 1981).

All of this research has shed a great deal of light on the neurobiological mechanisms which are necessary , specific and exclusive for learning and memory formation after training chicks on a passive avoidance paradigm. But if one can level any criticism, it would be the lack of follow-up experiments to further investigate a particular effect, so that the mechanism of change may be assesed.

It was with the aim of further investigating changes in glycoprotein metabolism with particular reference to the observed increase in fucose incorporation, as a result of training chicks on

P.A.L., that this thesis is based.

2.7 GLYCOPROTEINS

Classification

During the last fifteen to twenty years, glycoproteins have been recognized as an important group of compounds, and it is only with the development of modern analytical procedures that their structure and function have become more clearly elucidated (Clamp 1974). Glycoproteins can be defined in the simplest form as proteins with an attached oligosaccharide group, and have a wide variety of molecular sizes and structure. The reason for this heterogeneity in glycoprotein structure is due to the potential binding characteristics of each individual monosaccharide. For example, where as three identical amino-acids can adopt only one arrangement, three identical sugar molecules can have up to one hundred and seventy-six different arrangements! Glycoproteins range from globular proteins, such as ovalbumin, to very large extended molecules, such as the submaxillary glycoproteins, which differ mainly in their oligosaccharide or glycan content. Indeed, those glycoproteins which have a small proportion of saccharide as a prosthetic group are fairly ubiquitous, while those in which a heterosaccharide forms a large porportion of the molecule are found, in the main, in particular locations, such as cell surface, in connective tissue and in epithelial secretions. The simplest classification of glycoproteins is based on the type of covalent linkage which is formed between the carbohydrate moiety and the polypeptide chain. This falls into two categories (1) N-glycosidic linked and (2) O-glycosidic linked. The N-glycosidic linked glycoproteins contain oligosaccharides linked to the amide nitrogen of asparagine via a glycosylamine bond; examples are fetuin and thyroglobulin. O-glycosidic linked glycoproteins are formed by a bond between N-acetylgalactosamine to serine or threonine, and are

arbitrarily called mucin-type glycoproteins as they are usually found in this type of proteinaceous material (Spiro et al 1974). Examples of the structural formulations of O- and N- linked glycoproteins are given below Figure 2.2.

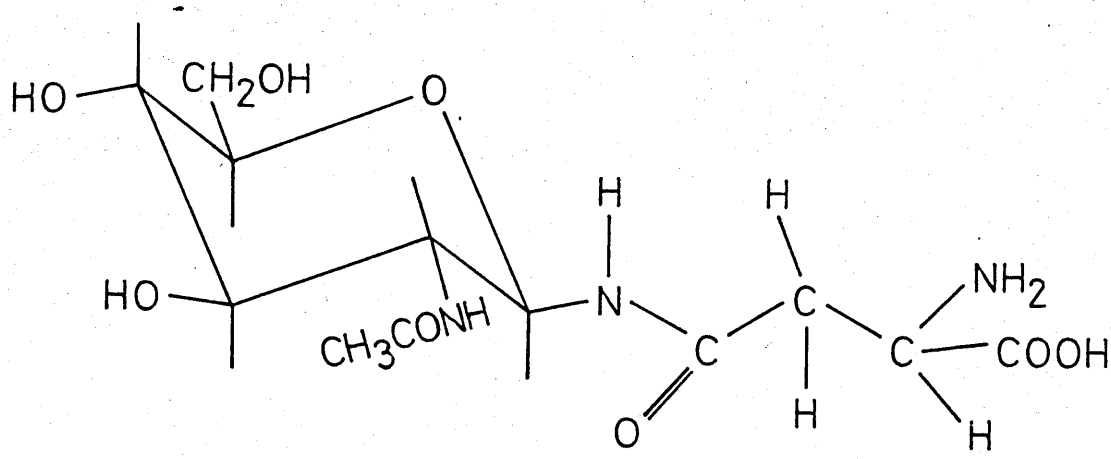
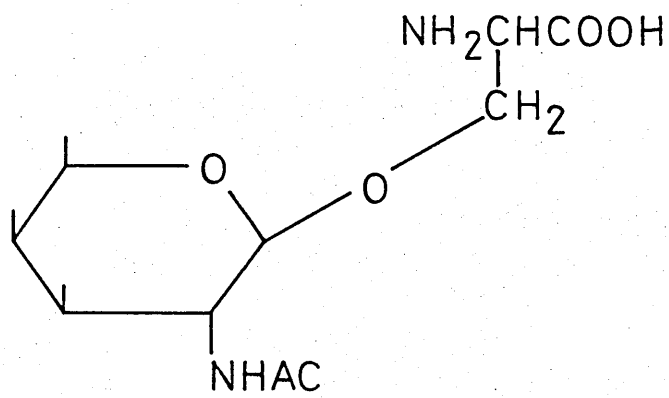


Figure 2.3. The structure of linkages present in N-linked glycoprotein, showing a linkage between N-acetylglucosamine and asparagine (above). The asparagine forms part of an extended polypeptide sequence and the N-acetylglucosamine residue forms the point of attachment for other monosaccharides. For convenience, the hydrogen substituents of the sugar ring are omitted.



The structure of linkages present in O-linked glycans, showing a linkage between N-acetylgalactosaminyl and serine (above). The sugar is linked by C (1) glycosidic bonds to the hydroxylated side chains of serine.

Synthesis

Understanding the glycosylation process and the subcellular sites involved is a necessary prerequisite before one can discuss possible regulatory factors which may influence glycoprotein production and the role the carbohydrate moiety plays in determining biological function

Assembly of the saccharide unit is believed to occur upon completion of the synthesis of the peptide chain, which occurs on bound polyribosomes (Spiro & Spiro 1966). After release of the nascent peptide from the polyribosomes into the channels of the endoplasmic reticulum, the bulk of the carbohydrate portion is added to the peptide back-bone, mediated by glycosyltransferases. The carbohydrate portion is initially assembled on a lipid carrier, dolichol pyrophosphate, a complex polyisoprenoid which acts as an anchor for the successive addition of activated sugars (by specific cytosolic kinases) to form the oligosaccharide. On completion of the oligosaccharide, it is transfered 'en-bloc' to the polypeptide backbone via a N- or O-glycosidic linkage, with the elimination of the lipid which probably returns to the dolichol pool by dephosphorylation (Waechter & Lennarz 1976). After transfer to the polypeptide, which passes into the Golgi apparatus, extensive modification of the oligosaccharide occurs in a complex series of reactions known as 'processing'. These involve removal of specific monosaccharides by glycosidases, in particular glucose and mannose residues. At present, little is known about the factors controlling processing and how the exact sequence of removal of mannose and glucose residues takes place. Variation of processing in different cell types probably leads to discrete glycan chains that are characteristic of those cells (Hughes 1983). The final step in glycoprotein formation is the termination sequence, in which the terminal sugar is enzymatically transferred

directly from the nucleotide derivative via a specific membrane-bound transferase to the chain. The most common terminal amino-acids are sialic acid (which is a substituted neuraminic acid derivative), mannose and fucose. Depending on the type of the glycoprotein it will be synthesised either totally within the cisternae of the membrane system and secreted from the cell (by vesicular formation of the golgi and exocytosis) or it will remain part of the golgi membrane structure and will eventually be incorporated into the plasma membrane (reviewed by Snider 1982).

Figure 2.3 outlines the process of glycosylation and the localization within the cell.

Only limited information is available on the factors which control glycoprotein synthesis. Whereas genes control the assembly of peptide chains by an accurate template mechanism, the synthesis of oligosaccharide prosthetic groups is controlled by a non-template mechanism. Initiation, elongation and possibly termination of the oligosaccharide appear to be controlled by the substrate specificities of these transferases. Thus, every oligosaccharide prosthetic group is assembled by the concerted action of a multiglycosyltransferase system (Schater & Roden (1973).

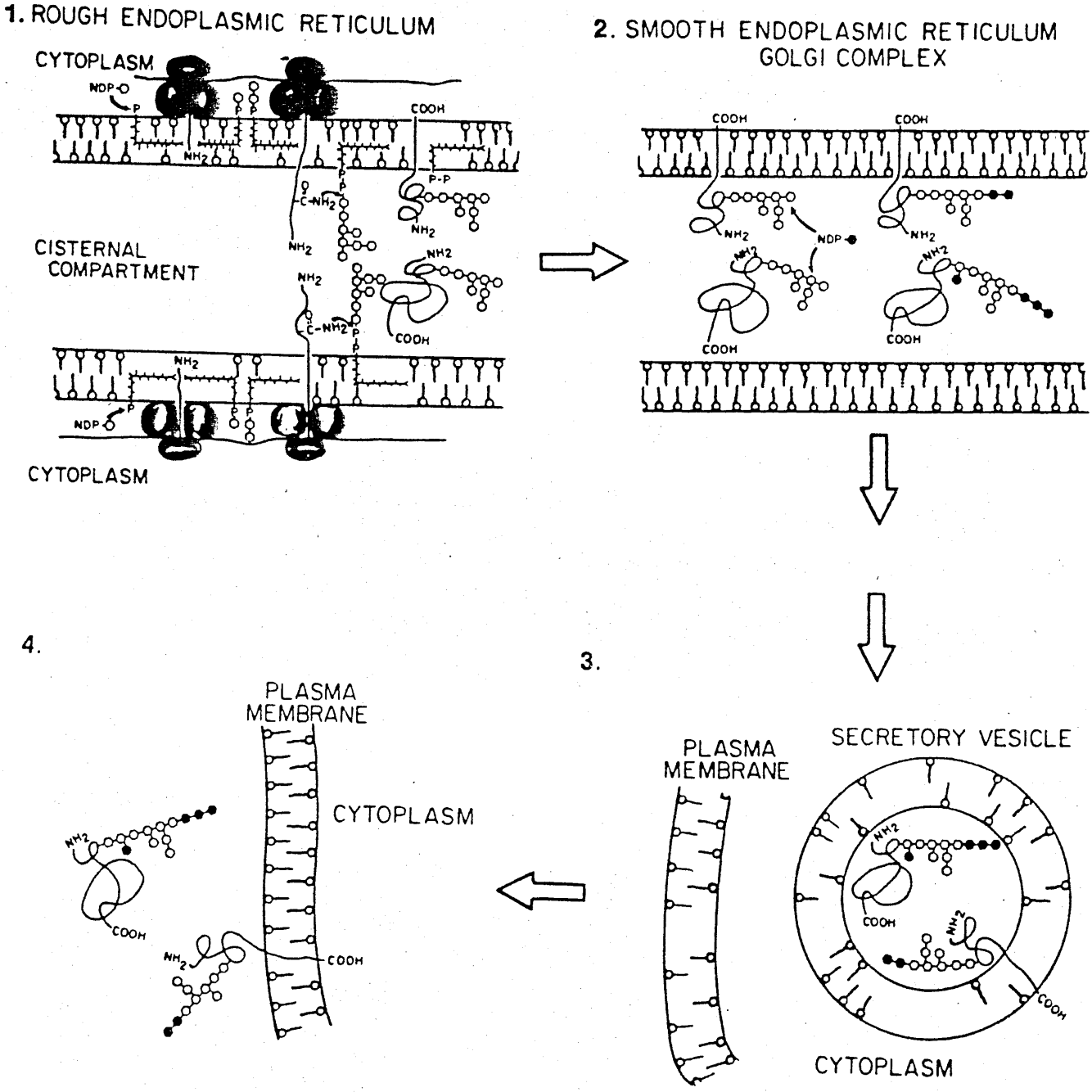


Figure 2.4. This Figure depicts the synthesis of secretory and membrane glycoproteins. 1: the oligosaccharide chain is transferred to the protein moiety. 2: the chain is 'trimmed' and the terminal sugar added. 3: the glycoprotein is bound in a secretory vesicle. 4: the vesicle is moved to the cell membrane, with which it fuses.

Functions

There are basically two types of glycoprotein, secretory and membrane bound, and they have many diverse functions. The actual glycan structure is vital for glycoprotein function, as studies involving the inhibition of glycosylation have shown that there is marked reduction in biological activity with consequent proteolysis of the naked protein and aggregation in the endoplasmic reticulum (Schwartz & Datema 1982).

Secretory glycoproteins include a range of enzymes, hormones and immunoglobulins which all play critical roles in cell function, but my main concern here is with membrane bound glycoproteins.

Membrane glycoproteins are orientated so that the sugar chains protrude from the outer surface of the membrane and as such this critical location endows the carbohydrate moiety with the following main properties.

1. Biological Recognition. The common molecular basis underlying the function of the carbohydrate in biological recognition is the interaction of the polysaccharide portion with polysaccharides, for example, the chain-chain interactions of many linear carbohydrates or the interaction of carbohydrate-binding proteins (e.g. lectins). The interactions may be between molecules integrated into the cell surface membrane of adherent cells, or may be mediated by aggregation factors. In the latter case, the aggregation factors may be products secreted into the intracellular space by either interacting cell or by some cell remote from the site of specific cell-cell adhesion. This cell would then have an inductive effect on the aggregation of other cells, a phenomenon very often encountered in tissue differentiation. Examples of glycoproteins which mediate specific cell-cell adhesion interactions are N-CAM - neural cell adhesion molecule (which has

recently been confirmed to be the same molecule previously called D2 and BSP by different groups), and N-gCAM - neural glial cell adhesion molecule (Edelman 1984). Other evidence of the cell recognition properties of glycoproteins is the antigenic properties of cells on which the whole study of immunology is based.

2. Stabilizing Biological Membranes. Cell surface glycoproteins possibly act as structural components to stabilize cell membranes by their ability to influence protein folding and conformation (particularly in bacterial cells). A major short- coming, however, in experiments designed to demonstrate a structural role for animal cell surface glycoproteins is the fact that many of these glycoproteins may be involved in interactions with cytoskeletal components at the inner surface of the membrane. Thus, changes in cell shape, elasticity, mobility, etc, following alterations of surface glycoproteins cannot necessarily be attributed to the altered glycoprotein structure, but may result from concomitant changes in the cytoskeletal system (Berger et al 1982).

The inherent polymorphism of the glycoprotein molecule (the many possible permutations and combinations in the chemical structure and configuration of the polysaccharide chains attached to the protein molecule) and its strategic position in the plasma membrane suggest that glycoprotein molecules may be candidates involved in changes in the functional connections of neurones. It is thus tempting to speculate that they may be involved in neurobiological mechanisms that mediate the storage, consolidation and retrieval of information, that is, learning and memory.

Glycoproteins in Learning and Memory.

Brain tissue contains a high concentration of glycoproteins; it has been calculated that as much as five to fifteen per cent of the total protein content of this tissue may consist of glycoproteins and approximately eighty per cent of these are located in the microsomal fraction and synaptic membranes (Brunngraber 1969, 1972). Forman et al (1972), using intracerebral injection of labelled fucose and subsequent autoradiographic analysis of tissue fractions at various times after injection, have revealed the localization of glycoprotein synthesis. They have determined that glycoproteins are synthesised in the cell body and rapidly transferred along the axon to the nerve ending. One fraction accumulates mainly as components of synaptic vesicles and the pre-synaptic plasma membranes, and another participates in renewal of axolemmal glycoproteins.

In order to monitor changes in glycoprotein metabolism, many researchers have used either labelled glucosamine or fucose, as these are predominantly found as terminal sugars in the oligosaccharide chain and are therefore very accessible to experimentation. However, the metabolism of glucosamine is complex, with possible conversion into N-acetyl glucosamine, N-acetyl galactosamine and sialic acids, and possible incorporation into sulphated acid mucopolysaccharides as well as heteroglycoproteins, glycolipids and gangliosides (Barondes 1968). Consequently, many investigators in this field have opted to work with fucose due to its specific metabolism in brain and most other tissues. Fucose, a six carbon structure containing a methyl group endowing it with lipophilic properties (Brunngraber 1969), is not catabolized. It is incorporated almost exclusively into glycoproteins as fucose and is not found in appreciable amounts in any other constituents, including glycolipids and acid-mucopolysaccharides (Margolis & Margolis 1972). The specific metabolism of fucose has

been investigated in the rat by Bekesi & Winzler (1967) and Bocci & Winzler (1969). After a six hour period, the metabolic fate of a dose of L-^{14}C-fucose was traced; 1.6% of the total dose appeared as CO_2, 30% was found in urine as free fucose and the remainder was found in tissue and serum glycoproteins.

The structure of L-fucose is shown below in Figure 2.5. It differs from other sugars found in mammals because of its L-configuration and the absence of an hydroxyl group on carbon six.

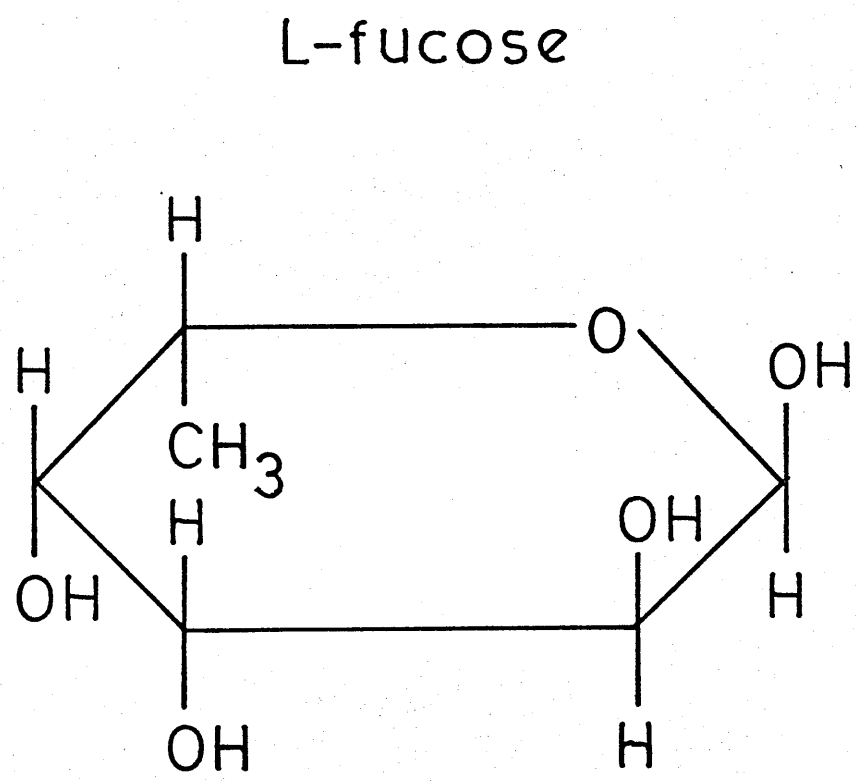


Figure 2.5 Structure of L-fucose.

As previously mentioned, fucose occupies a terminal position on the carbohydrate chain of glycoproteins, thus making it a particularly useful tool for studying changes in glycoprotein metabolism during

development and, more specifically, their involvement in mediating neuronal changes (Ginsburg & Neufield 1969; Zatz & Barondes 1970).

Studies to determine the basic metabolism of fuco-glycoproteins in the rat and mouse brain have been performed by Quarles & Brady (1971), Zanetta et al (1977) and Zatz & Barondes (1969). After intracranial injections of labelled fucose and subsequent fractionation of the brain, labelled fuco-glycoproteins were extracted. Fuco-glycoproteins had a slow turnover rate (in mouse, the half-life was found to be four weeks) and the concentration of total fuco-glycoproteins increased about three-fold between six and twenty-nine days post-natal age. After an intraperitoneal injection in young rats, maximum incorporation of tritium-labelled fucose into brain glycoproteins occurred after 3-6 hours, and the half-life of fucose containing glycoproteins was 2 weeks (Margolis & Margolis 1972). The composition of the carbohydrate portion is altered (Dibenedeppo & Cioffi 1972), with a greater rate of turnover of this portion in the young rat. Subcellular fractionation studies revealed that most of the labelled fucose was incorporated into the P2 fraction (containing myelin fragments, synaptosomes and mitochondria) and the microsomal fraction, containing both smooth and rough endoplasmic reticulum and other membrane fragments; these are the major sites of glycoprotein synthesis, as discussed previously (see also Zatz & Barondes 1970). Electrophoresis of membrane bound labelled glycoproteins on polyacrylamide gels indicated that they were extremely heterogenous, with apparent molecular weights ranging from 30,000 to 250,000; the highest concentration of label occurred in glycoproteins with molecular weights of between 40,000 and 70,000 (Quarles & Brady 1971).

In view of the accumulating evidence of the importance of membrane glycoproteins, many researchers have looked at the role they may play in learning and memory processes, using fucose as a marker. In an attempt to examine whether L-fucose incorporation into hippocampal glycoproteins would be a suitable tool for studying such a process, Popov et al (1976a) conducted an extensive biochemical and histoautographic study. They found that after an intraperitoneal injection of radioactive fucose, there was a rapid decrease in labelled fucose in the soluble portion and a rapid deposition into organs (spleen and kidney). They found that there was only a small pool of free intracellular fucose present, amounting to 0.02 umol/gm tissue, whereas the content of bound fucose was 0.8 umol/gm tissue. This was an important finding, as future experimental calculations could therefore legitimately ignore the concentration of free intracellular fucose (this assumption has also been applied to experimental results reported in this thesis).

Damstra-Entingh et al (1974) and Damstra et al (1975) have reported an increased incorporation of labelled fucose into glycoproteins of mouse brain and liver as a result of being exposed to an enriched environment, the training apparatus. The observed changes were thought to be related to certain types of sensory input. Further studies by Popov et al (1976a, 1976b, 1980), showed that after training rats on a brightness discrimination task, there was an increase in fucose incorporation in trained rats over controls. This increased incorporation was localized in the hippocampus, with a smaller increase occurring in the visual cortex. Electrophoretic analysis of the labelled glycoprotein showed that particular glycoproteins in the C.A 1 region of the hippocampus showed an increased labelling at certain times before and after training (Popov et al 1982). Subsequent studies showed that this increased fucose

incorporation could be elicited in the hippocampus *in vitro* upon electrical stimulation (Jork *et al* 1979). There is good agreement between these studies and those of Sukumar *et al* (1980), which indicated an increase in fucose incorporation into particulate proteins as a result of training chicks on a passive avoidance paradigm.

The attention of both groups was subsequently turned to examining the possible mechanism of increased fucose incorporation. Studies indicated that in both the chick and the rat (after training on their respective paradigms) there was an increase in fucokinase activity, an enzyme involved in the incorporation of fucose into glycoproteins (Popov *et al* 1983; Lossner & Rose 1984).

So evidence strongly indicates that changes in glycoprotein metabolism, as investigated using the specific terminal sugar fucose, are indeed biochemical events directly related to training (learning) and engram formation (memory).

This is the point at which this thesis takes up the further investigation of the importance and implications of the observed changes in fucose incorporation as a result of training chicks on a passive avoidance learning paradigm. Initially, I further investigated the properties of the chick fucokinase enzyme in order to locate specific intracellular and/or extracellular factors which may influence activity (Chapter 3). Subsequently, I developed an *in vitro* fucosylation system, with the ultimate (and rather ambitious) aim of locating and identifying specific glycoproteins which are critical to learning and memory (Chapter 4). Finally, I devised a series of experiments to determine if the observed increase in fucose incorporation was an independent post-translational process or if it was coupled to an increase in protein synthesis *de-novo* (Chapter 5).

CHAPTER 3

FUCOKINASE ACTIVITY IN THE CHICK FOREBRAIN.

Given the history of research on increased fucose incorporation as a result of training discussed earlier, my initial experiments were concerned with the mechanisms by which this increase may be elicited.

Stimulated by the observations of Lossner & Rose (1983) that on training chicks on P.A.L., there was an increased fucokinase activity in (M) trained birds over (W) control birds one hour after training, I decided to follow this line of investigation. Fucokinase, one of the enzymes in the metabolic pathway of fucose incorporation, is easily isolated and its activity assayed. My initial experiments were concerned with the further characterisation of the properties of chick brain fucokinase, its developmental and diurnal profile and subsequently a time course of activity change at various times after training.

Fucokinase (ATP;6-deoxy-L-galactose 1-phosphotransferase, E C 2.7.1.52) is one of the enzymes involved in the activation of fucose before its incorporation into glycoproteins. It has been suggested that fucokinase is found in all tissues in which there are L-fucose containing glycoproteins, including porcine liver (Ishihara et al 1968; Yurchenco & Atkinson 1975), porcine submaxillary glands (Richards et al 1978), canine thyroid tissue (Richards & Serif 1977)

and rat and chick brain (Popov et al 1983; Lossner & Rose 1983). Fucokinase is the enzyme responsible for the phosphorylation of fucose to form fucose-1-phosphate, which is subsequently activated to form the nucleotide GTP-fucose by fucose pyrophosphorylase. It is in this form that fucose is transferred, via a specific fucosyl-transferase, directly to the terminal position of a growing glycan chain to form a complete fucose containing glycoprotein. This is the mechanism by which dietary and recycled fucose is utilised in mammalian tissues (Ishihara et al 1968); however an alternative pathway of GDP-L-fucose formation is probably via glucose ------ glucose-6-phosphate ------ fructose-6-phosphate ------ mannose- 6-phosphate ------ GDP-mannose------ GDP-4-keto-deoxy-mannose ------ GDP-L-fucose, if there is no free fucose available (Ginsberg 1960; Foster & Ginsberg 1961).

The enzymatic pathway of fucose incorporation into glycoproteins and the location of each enzyme within the cell is outlined in Figure 3.1.

The fucokinase assay used in the work reported in this thesis was initially described by Ishihara et al (1983) for pigeon liver tissue and was subsequently modified for chick brain by Lossner & Rose (1983). This assay, which is based on the measurement of fucose-1-phosphate formation using labelled fucose (L-(^{14}C)-fucose), revealed that the optimal parameters of incubation were a temperature of 37 C for a period of 60 minutes, with a protein concentration of 150 - 800 ug. The pH optimum for enzyme activity was approximately 7.5, using a concentration of fucose of 5 x 10^{-5} M. The recorded values for Km and Vmax for chick fucokinase were 4.5 x 10^{-6}M and 3.72 nmoles/mg protein/ hr, respectively. Using these observations and essentially the same assay procedure, I started work.

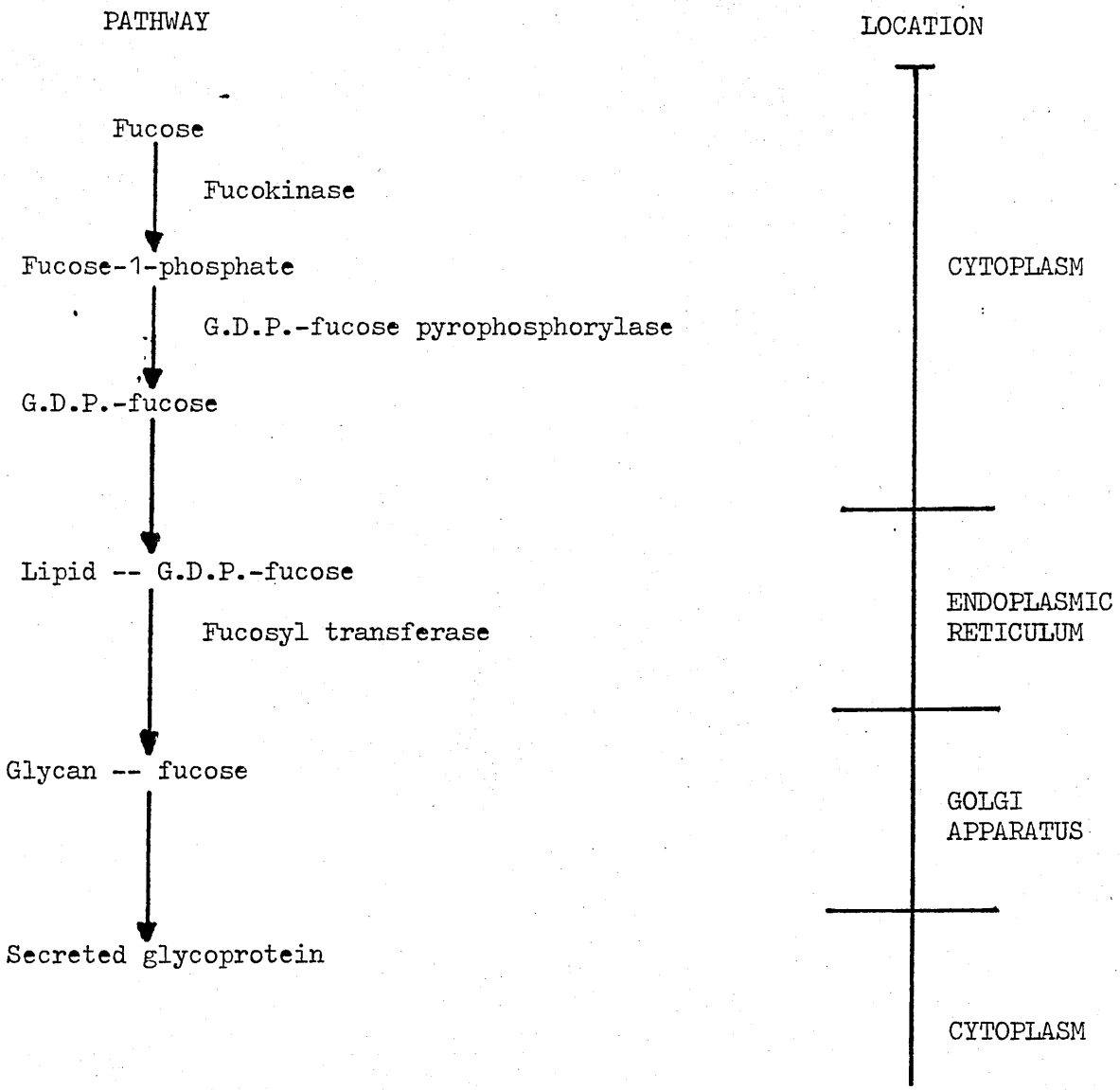


Figure 3.1. The enzymatic pathway of fucose incorporation into glycoproteins, showing the location, within the cell, of each enzyme involved. See text for further explanation.

3.1 MATERIALS AND METHODS

Dissections

In all experiments, dissections of the chick forebrain were carried out using an araldite mould with which one could could obtain reproducible forebrain base and roof regions. These dissections were similar to those of Bateson et al (1969), as drawn in Figure 2.1. Subsequently, the hemispheres were separated to give four distinct regions: left roof (L.R.), right roof (R,R,), left base (L.B.) and right base (R.B.).

Biochemistry

Chick brain tissue (approximately 3 mg of protein) was homogenized with a Polytron homogenizer (setting 4) in 1.5 ml 77.5 mM Tris-HCL buffer, pH 7.5. Included in the buffer was 130 mM KF (to prevent further phosphorylation reactions) and 3mM dithioerythritol. The tissue was then centrifuged at 40,000g for 45 min to isolate the Tris-soluble portion, which contained the fucokinase enzyme. All steps were performed at 4 C (on ice). 200 ul of the supernatant was then added to an incubation mixture of final volume 310 ul, containing 3.3 mM ATP/$MgCl_2$ and 0.05 mM L-(^{14}C)-fucose, specific activity 29.6 MBq/mmol (Amersham International), diluted with cold fucose to a final activity of 2.22 GBq/mmol. After an incubation period of 45 min at 37 C, the incubate was transferred to prepared activated resin in pre-made columns which immediately inhibited further enzymatic activity.

The columns were made from a pipette tip (Sterlin-Eppendorf-Blue) of volume 100-1000 ul, sealed with a cotton wool ball. After extensive washing of the resin (Dowex 2x8, 200-400 mesh), with 2M

NaOH, 2M HCL and finally water until neutral, each tip was filled to a bed volume of 0.4 ml. The purpose of the column was to separate unconverted (^{14}C)-fucose from (^{14}C)-fucose-1-phosphate, and this was achieved by initially activating each column with 10 ml of a solution containing 95g ammonium formate (1.5 M) and 22.13 ml formic acid (0.5 M). This procedure activates the resin with positive charge to which the phosphate groups bind. In order to elute all of the unconverted (^{14}C)-fucose, each column was washed with 6 ml 10 mM ammonium formate solution, until no radioactivity was detected in the eluate. The optimal flow rate was approximately 0.1 - 0.2 ml/min. Elution of the reacted (^{14}C)-fucose- 1-phosphate was accomplished by the addition of 2.2 ml of a mixture containing 0.2 M HCl and 0.2 M KCl. Controls for each experiment were samples kept at 4 C for the incubation period and analysed in the same manner as experimentals. Finally, 1.1 ml of the eluated fractions was added to 8 ml scintillation cocktail (Cocktail T, BDH Chemicals Ltd.) and counted at an efficiency of 80% in a Beckman scintillation counter. Experimental samples contained approximately 1000 dpm with controls/blanks registering less than 100 dpm. Differences in radioactivity reflected the amount of converted (^{14}C)-fucose-1-phosphate. Each sample was assayed in triplicate. Protein determination was by the method of Lowry et al (1951), using bovine serum albumin as standard.

Calculation of enzyme activity

Enzyme activity was calculated as nmole fucose-1-phosphate formed/mg protein/ hour. Assuming that the eluted fraction contains fucose-1-phosphate derived from fucose (15 nmole/sample) initially added (free intracellular fucose is negligible, as discussed in Chapter 2)) and provision is made for background corrections, activity is calculated as follows:

$$\frac{\text{dpm (eluate - blank) X 15 X 60}}{\text{dpm (standard - blank) X mg Prot. x 45}}$$

Standarization of results

When chicks from different hatches were used for a particular set of experiments, a standarization procedure was applied to the data in order to reduce variability arising from hatch differences (referred to in Chapter 2.). This standardization was justified by the experimental procedure which involved a balanced design of (M) trained and (W) control chicks, for all brain regions. This involved normalizing all the results around the means of each set of results obtained from chicks of different hatches. Statistical comparisons were made using an unpaired Student's t-test (two-tailed).

Developmental Study

A total of 72 female chicks from two hatches were used in this study, which involved the measurement of fucokinase activity in four brain regions (L.R., R.R., L.B. and R.B.) of chicks ranging in age from 24 hours to 35 days post- hatch. Activity was analysed at nine time intervals during this period and eight chicks were analysed at each time point. Chicks from both hatches were maintained under similar conditions (12 hour light/dark cycle and a temperature of

37 C) throughout the study and were killed at the same time of day. Before homogenization, the whole forebrain of each chick was weighed.

Diurnal Study.

Chicks, in an age range of 18 - 30 hours post-hatch from two hatches were killed at two-hourly periods over a period of 24 hr and fucokinase activity determined in whole forebrain. The two hatches were maintained under different conditions of photoperiod, one hatch was exposed to constant light, while the other was maintained on a 12 hr light/ dark cycle. The temperature was constant at 37 C for both hatches. The number of chicks from each hatch killed at each time point was 3 and 4, respectively.

Ion / Nucleotide Study

Studies to determine the effect of various concentrations of (1) calcium chloride ($CaCl_2$) (1mM - 5uM), (2) ethylene glycol tetra-acetic acid (EGTA) (10 mM - 0.5 um), (3) EGTA (10mM - 0.5 uM) and $CaCl_2$ (50 uM), (4) magnesium chloride ($MgCl_2$) (6.0 mM - 50 uM) and (5) manganese chloride ($MnCl_2$) (6.0 mM- 50 uM), on fucokinase activity were performed. An investigation to note the effects of two nucleotides was also carried to determine effects on control fucokinase activities. Various concentrations of cyclic adenosine mono-phosphate (C'AMP), ranging from 1 mM - 5 uM, and cyclic guanosine mono-phosphate (C'GMP), 1 mM - 10 uM, were investigated. For each ion and nucleotide studied, three experiments were performed and each concentration was studied in triplicate. 50 ul of each ion / nucleotide was added to the incubation medium and volumes of other constituents were reduced to maintain a constant original volume of 310 ul. In some experiments, 3.3 mM $MgCl_2$ was substituted with

various concentrations of $MnCl_2$. Each set of experiments was run in parallel with a control incubation, as indicated previously in the biochemical procedure, and enzyme activity obtained from each series of ion/nucleotide experiments was calculated as relative activity of that in the control incubation.

Time Course Of Activity After Training

Female chicks (18 - 30 hr post-hatch) were trained on the PAL paradigm (as described in Chapter 2) at the same time each day (08.00 hr). The train-test interval was varied from 1hr, 6hr, 10hr to 24hr. Immediately after testing, chicks which met the training criteria, (M) trained (chicks which had pecked the methylanthranilate coated bead and avoided on retest) and (W) trained (those that had pecked the water coated bead and pecked on retest) were killed and forebrains dissected into the four specified regions (L.R., R.R., L.B. and R.B.). The tissue was immediately transferred to pre-cooled tubes and placed on dry-ice. Samples were then analysed for fucokinase activity or stored at -16 C for a maximum period of 48 hr (after this time, there was marked reduction in enzyme activity). For this set of experiments, chicks were taken from a number of hatches and the number killed in each group, (M) trained (W) control groups at 1hr, 6hr, 10hr and 24hr was 15, 12, 10 and 10 respectively.

3.2 RESULTS

Developmental Profile.

As illustrated in Figure 3.2, fucokinase activity ranged from 3.24 - 5.75 nmoles- fucose-1-phosphate /mg prot/hr, an almost two fold increase in activity over the 35 day period. Activity increased up until day 20 and thereafter levelled off. Analysis of activity in the four forebrain regions showed that there were no significant differences in activity between regions, and so the graph indicates mean fucokinase activity values for the whole forebrain. Also included on the graph is a plot of total forebrain weight, which increased with age from 0.43 to 1.00 gram.

Diurnal Study

As indicated in Materials and Methods, two hatches were used in this study and were subjected to different light conditions. The sample sizes at each time point for each hatch were small (n=3 and n=4), due to small hatch size and the requirement of working with chicks of one sex. However, I have analysed the data using two methods, as shown in Figure 3.3 and Figure 3.4. Figure 3.3 shows the mean value of activity obtained at each time point as a percentage deviation from the mean value of total recorded activity for the 24hr period. Figure 3.3A shows the data obtained from the hatch maintained in constant light and Figure 3.3B shows data from the hatch subjected to a 12 hr light/ dark cycle. A legitimate statistical analysis can not be performed on the data as presented due to the small sample size, but from a visual inspection there seem to be no differences in fucokinase activity between the two hatches which could be attributed to different light conditions during the 24 hr period.

In order to perform a valid statistical analysis on this data, activity recorded from the two hatches was combined, such that n=7 at each time point. The was standardised (as described above) to eliminate hatch differences. Figure 3.4 shows the combined data of chick fucokinase activity over the 24 hour period. There are two peaks in activity, one at 11.00 hr and the other at 07.00 hr with a 30% difference between maximum and minimum activity levels. There is a significant difference in fucokinase activity over this period ($F=4$, $df=11,64$, $P<0.01$, one-way analysis of variance).

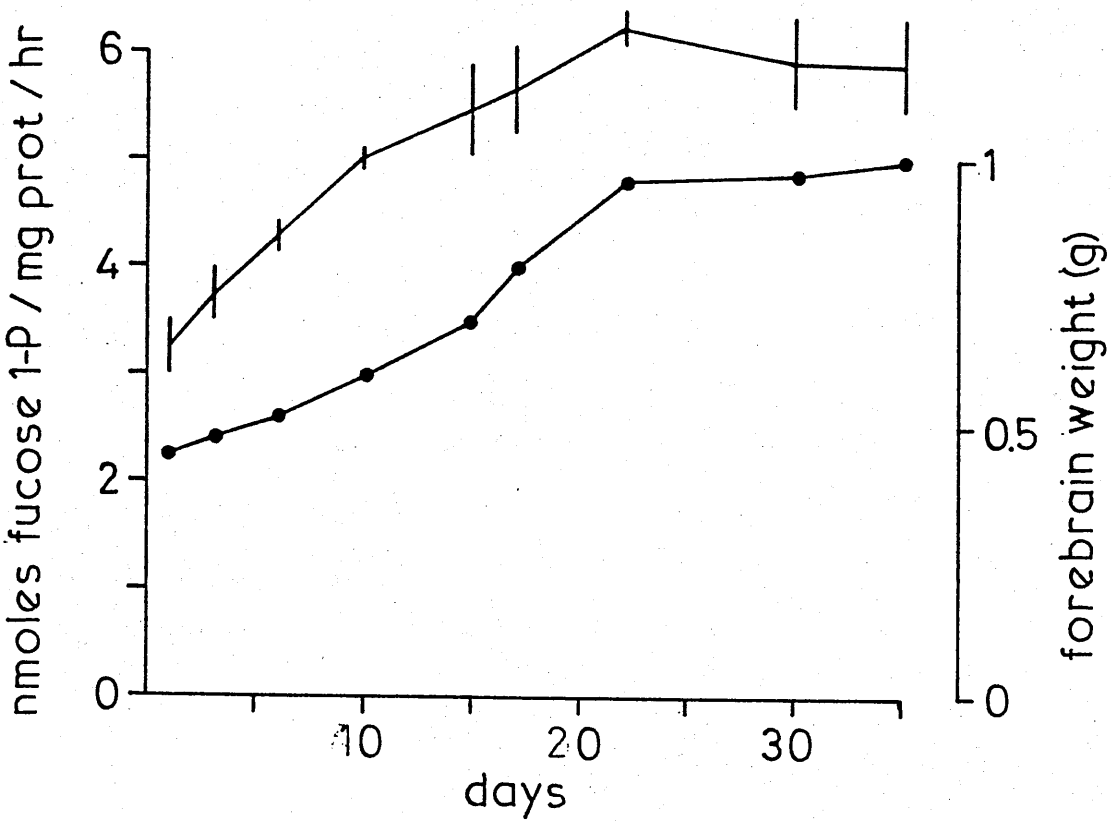


Figure 3.2. Chicks from two hatches were removed at various ages, ranging from 24 hr to 35 days. They were killed and fucokinase activity was assayed in whole forebrain as described in Materials and Methods. Each time point in the graph represents the mean ± SEM of fucokinase activity for eight chicks. Before the assay, the weight of whole forebrain was measured (filled circles in the graph).

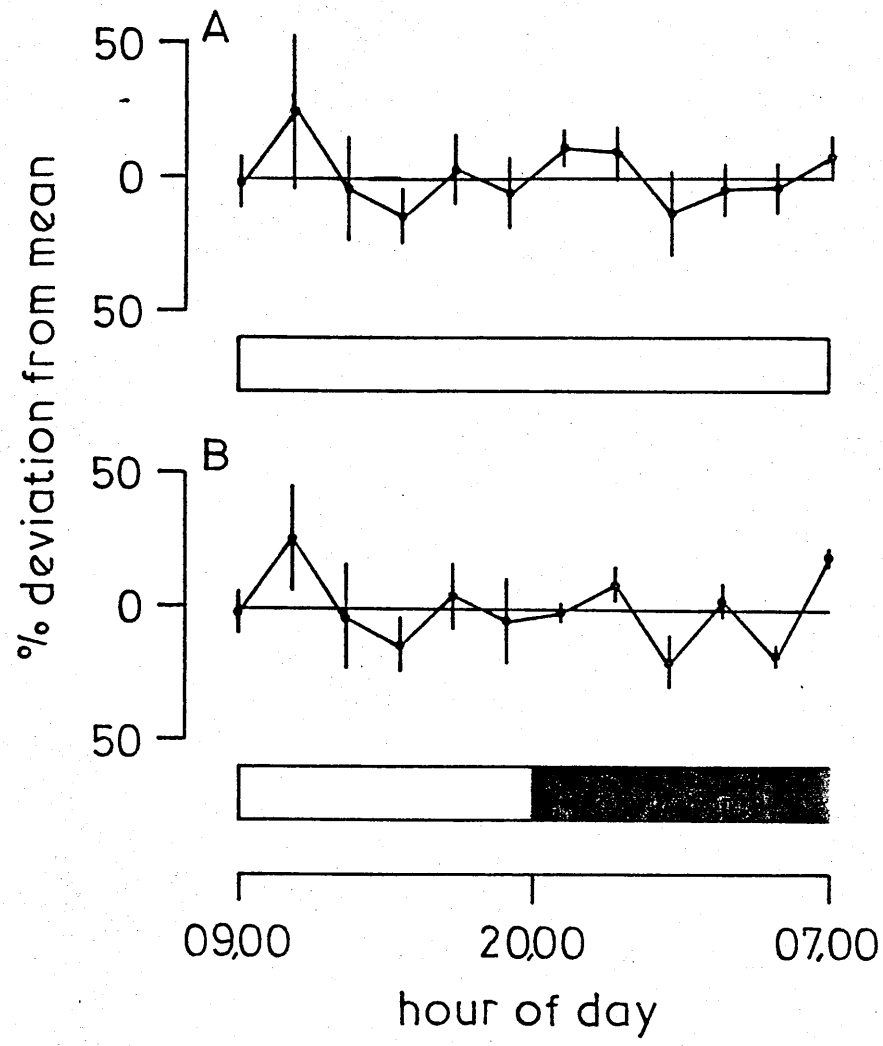


Figure 3.3. Chicks were taken from two different hatches at intervals of 2 hr, were killed and fucokinase activity in whole forebrain assayed as described in Materials and Methods. This Figure shows the percentage deviation (± SEM) from the mean value of all recorded activities of each hatch. In A, fucokinase activity in chicks maintained under constant light for 24 hr is shown; three chicks were killed at each time-point. In B, fucokinase activity in chicks maintained on a 12L:12D light-dark cycle is shown; four chicks were killed at each time-point.

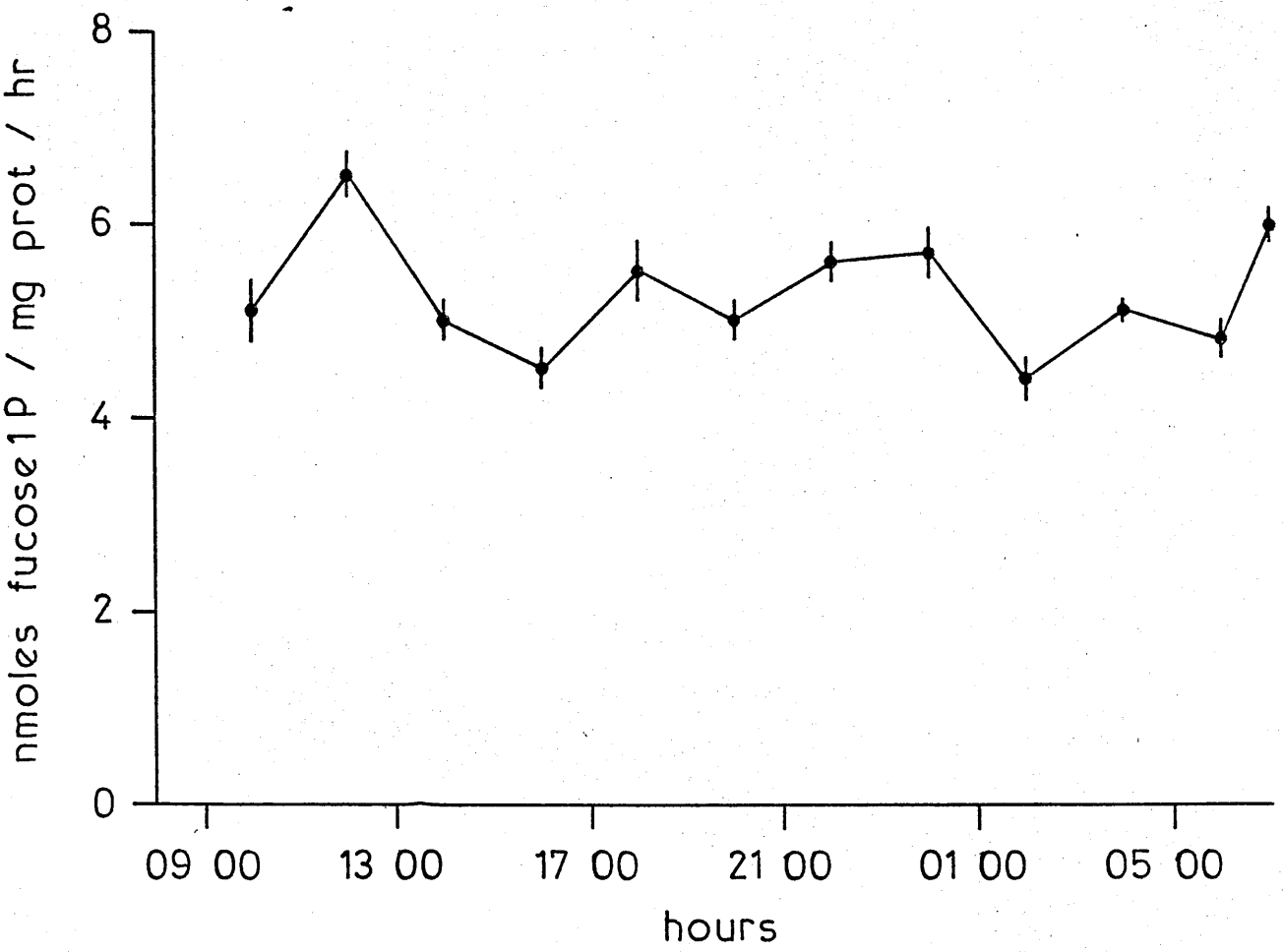


Figure 3.4. In this graph, the data presented in Figure 3.3. have been combined; n = 7 at each time-point. The graph shows changes in fucokinase activity over a period of 24 hr. There are two peaks in activity, one at 11.00 hr and the other at 07.00 hr. There is a significant difference in activity over the 24 hr period ($F = 4$, df = 11, 64, P 0.01, ANOVA).

Ion/ Nucleotide Study

When Ca^{2+} was added to the incubation medium in concentrations ranging from luM - 1mM, there was a marked inhibition of enzyme activity, with the highest concentration of the ion (1 mM) causing a 65% inhibition of control activity (Figure 3.5). The Ic_{50} for Ca^{2+} giving half maximal inhibition of fucokinase activity was 50 uM.

To determine the effects of varying the concentration of magnesium present in the incubation medium, a range of concentrations of this ion was studied. Figure 3.6 shows that activity increases with increasing concentration up to 3.3 mM, at which maximum fucokinase activity is obtained. This is the concentration which is used in the original (control) assay procedure. With higher concentrations, activity was reduced.

Similar experiments were conducted to investigate the effects of manganese on activity. When various concentrations were added to the incubation medium in the presence of 3.3 mM $MgCl_2$, no effects on activity were noted. However, when $MgCl_2$ was substituted with $MnCl_2$, a concentration of 0.5 mM gave maximum fucokinase activity, which was 60% of the activity obtained under control conditions (Figure 3.7).

When the effect of the nucleotides C'AMP and C'GMP were investigated, an inhibition in enzyme activity was noted with the highest concentration 3.3 mM, giving a 40% and 20% inhibition of control activity, respectively (Figure 3.8).

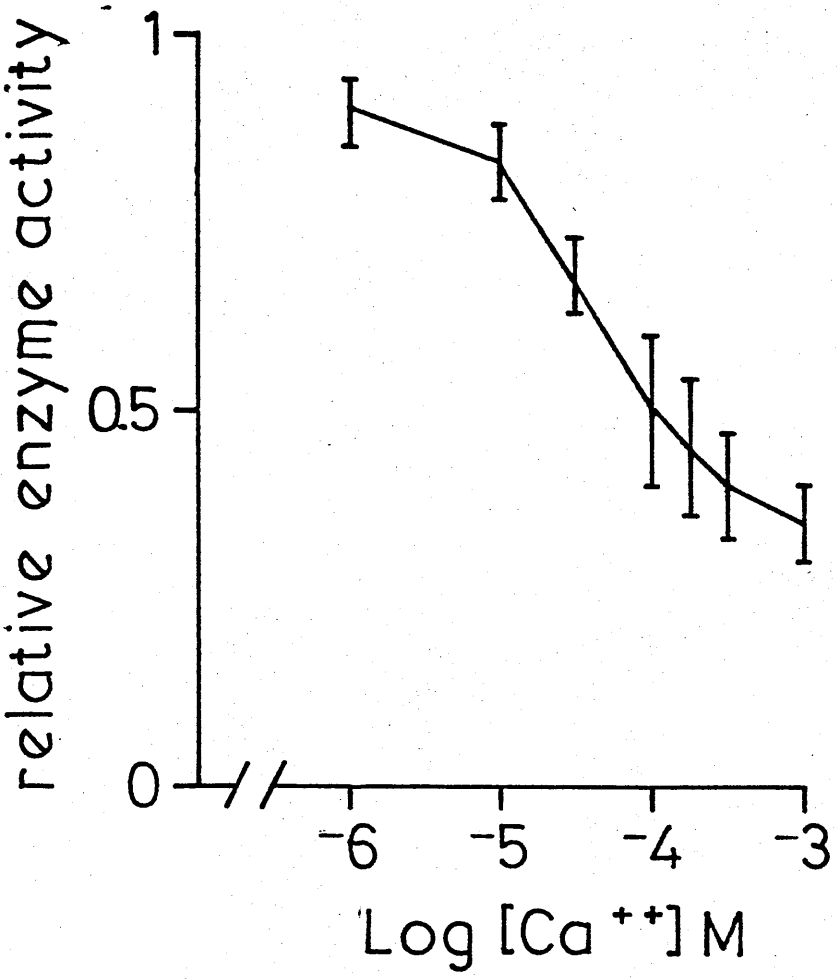


Figure 3.5. Whole forebrain was homogenized, as described in Materials and Methods. The supernatant (3 mg protein) was incubated as described with the addition of various Ca^{2+} concentrations, ranging from 0.1 um to 1.0 mM. This graph shows the relative enzyme activity (of control activity), for each concentration (control activity, with no Ca^{2+} present, is designated as 1.0). Each value represents the mean $\pm$ SEM of three separate determinations.

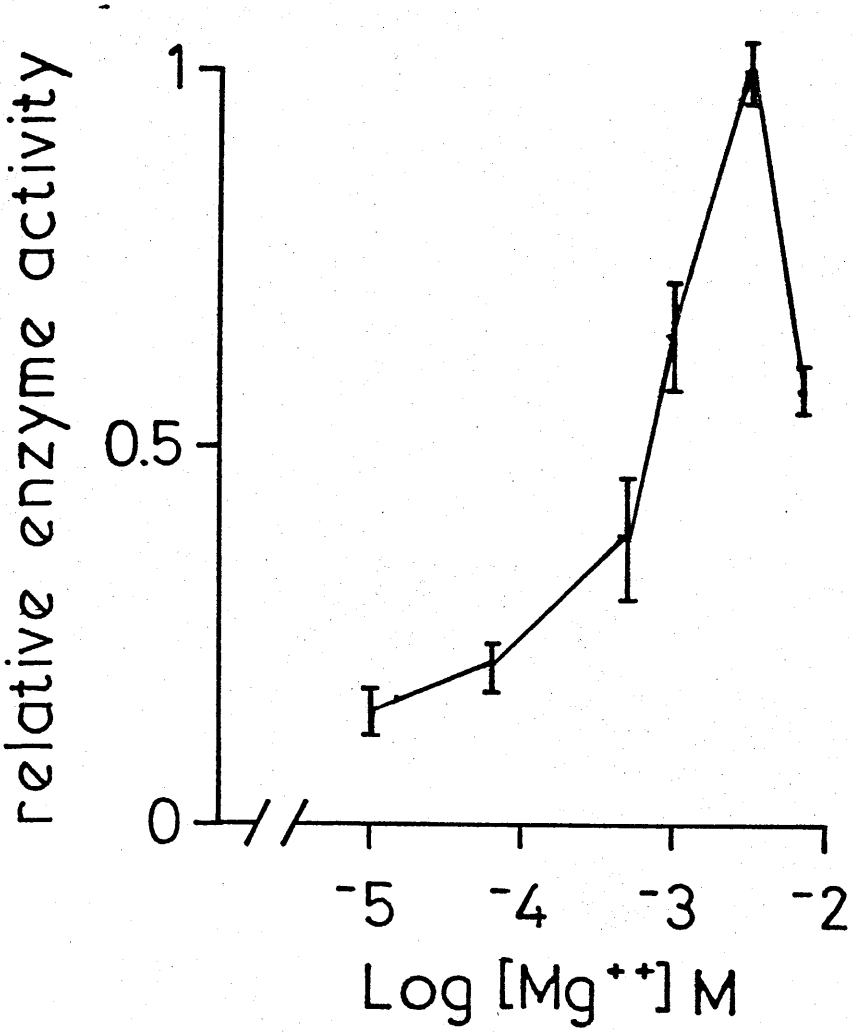


Figure 3.6. Whole forebrain was homogenized, as described in Materials and Methods. Aliquots of the supernatant (3 mg protein) were incubated as described with the addition of various Mg^{2+} concentrations, ranging from 10 um to 10 mM. This graph shows the relative enzyme activity for each concentration. Control activity, designated as 1.0, was determined at a Mg^{2+} concentration of 3.3 mM.
Each value represents the mean $\pm$ SEM of three separate determinations.

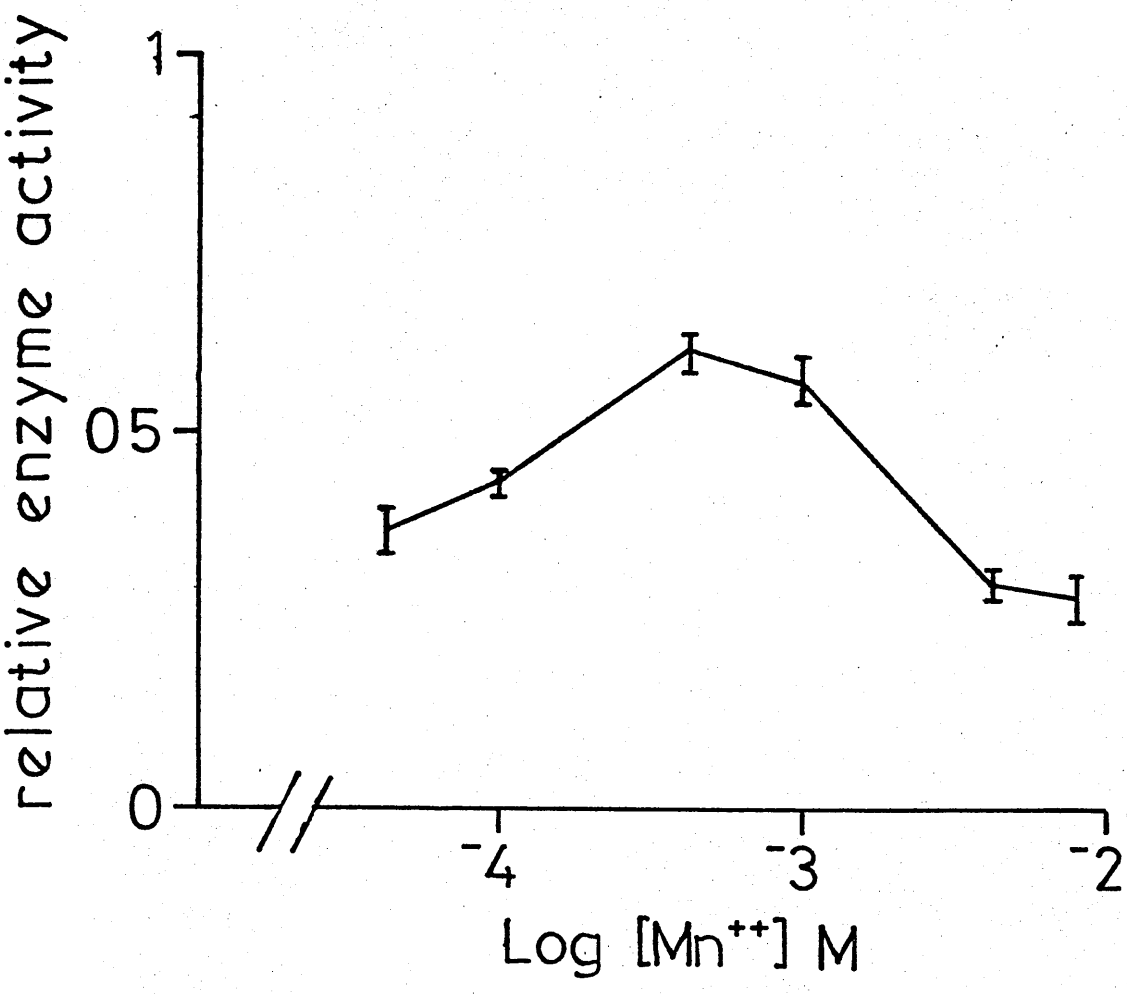


Figure 3.7. Whole forebrain was homogenized, as described in Materials and Methods. Incubation was as described, except that 3.3 mM Mg^{2+} was replaced with various concentrations of Mn^{2+} (50 uM to 6.6 mM). This graph shows the relative enzyme activity for each concentration; control activity, designated as 1.0, was determined at a Mg^{2+} concentration of 3.3 mM. Each value represents the mean $\pm$ SEM of three separate determinations.

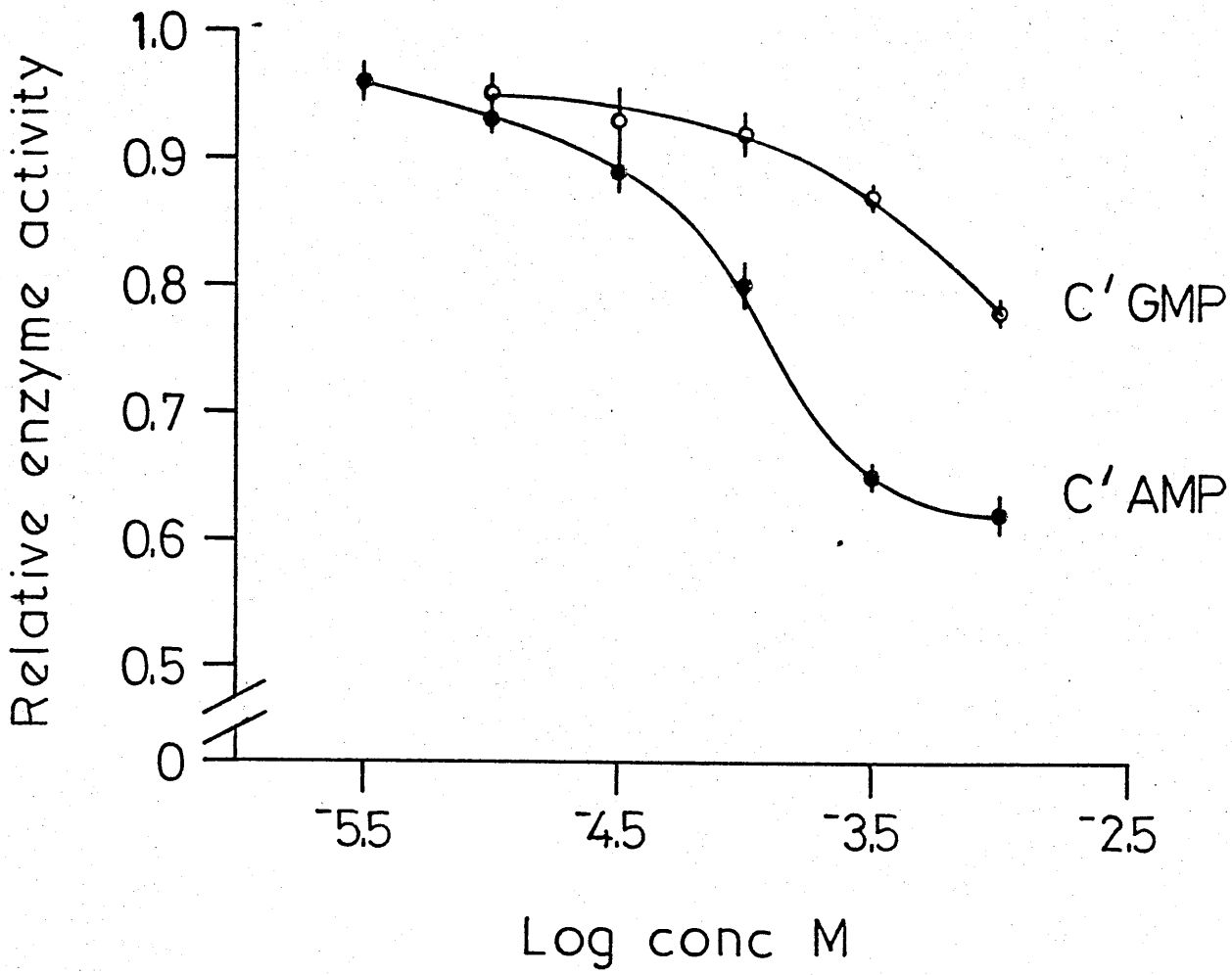


Figure 3.8. Whole forebrain was homogenized and the supernatant incubated as described in Materials and Methods, but with the addition of a range of concentrations of c'AMP (2.5 uM to 1.0 mM) or c'GMP (5 uM to 1.0 mM). Each concentration of nucleotide was studied in three separate experiments and fucokinase activity determined. Activity was expressed relative to control activity under normal incubation conditions. Values represent mean $\pm$ SEM of three separate determinations.

Time Course Of Fucokinase Activity After Training.

Figure 3.9 shows the activity of fucokinase at 1hr, 6hr, 10hr and 24hr after training. Activity was investigated in the four specified regions of the forebrain in both (M) trained and (W) control birds. One hour after training, a significant increase of 15% ($t = 3.52$ $P<0.05$, two-tailed t-test) in activity was observed in the left forebrain base of (M) trained birds over (W) birds. Six hours after training, a significant increase in activity of 20% was found in the right forebrain roof ($t = 2.08$ $P<0.05$, two-tailed t-test) in the (M) birds over the (W) controls. No difference in activity was noted in any other region at these time points, and no change in activity was observed in any region when birds were killed ten hours and twenty-four hours after training. It is important to notice that statistical analysis was performed on each set of data obtained at specific times after training independently. The absolute values of fucokinase recorded varied from the lowest recorded value of 2.08 at 1 hr to the maximum value of 4.52 nmoles fucose-1-phosphate/mg prot/hr at 24 hr.

I also carried out training and biochemical analysis (fucokinase activity) with chicks which were 12-24 hr posthatch. Although the percentage of chicks which met training criteria for both the (M) trained and (W) control groups was comparable to that obtained for the 24-30 hr post-hatch animals, no change in fucokinase activity in any region one hour after training was found.

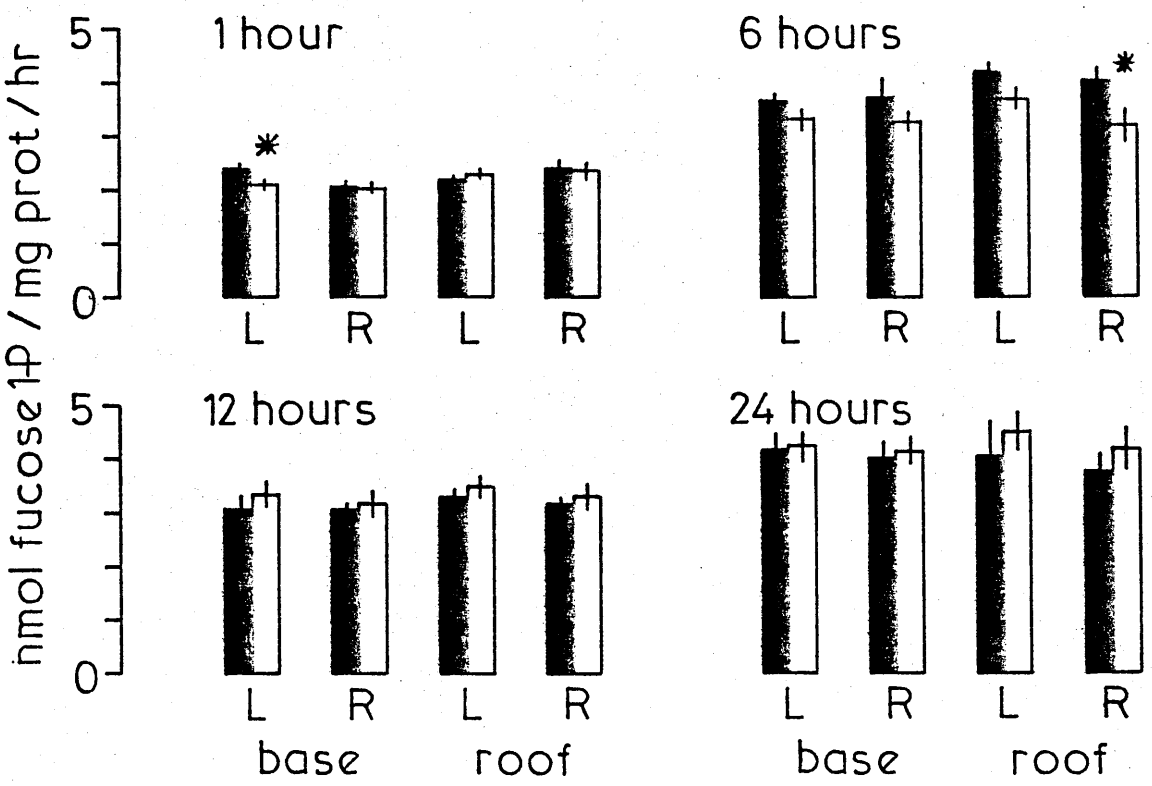


Figure 3.9. Whole forebrains from trained and control chicks were divided into four distinct regions: left (L) base, right (R) base, left (L) roof and right (R) roof. Each region was homogenized as described in Materials and Methods, and fucokinase activity was determined. This Figure shows activity in trained (filled columns) and control (open columns) chicks at intervals of 1 hr, 6 hr, 12 hr and 24 hr after training; n = 10 in each group. There is a significant increase in activity in left base of trained chicks over controls 1 hr after training ($t = 2.19$, $P < 0.05$). Six hours after training, activity is significantly greater in the right roof of trained chicks ($t = 2.13$, $P < 0.05$).

3.3 DISCUSSION

The initial studies of Lossner & Rose (1983) on chick fucokinase activity indicated that the enzyme had very different properties to those observed for fucokinase in other tissues, including HeLa Cells (Yurchenko et al 1975) and dog thyroid (Richards et al 1977). The observed Km value of chick fucokinase is 4.2 uM (approximately 6 - 25 fold lower), while the estimated Vmax of 3.72 nmol/mg prot/hr is much higher than that recorded for other tissues. This may be a consequence of the rate of turnover of fucose-containing glycoproteins in the chick brain, although this has not been investigated. Zatz & Barondes (1970), in experiments with mouse brain, showed that fucoproteins had a slow turnover rate and increased in total amount about three- fold between six and twenty-nine days post-natal age. However, Glasgow et al (1972) and Muller et al (1985) have shown that there is a decreasing trend in incorporation of fucose into mouse and rat brain glycoproteins respectively, within 2 - 30 days of post-natal life, with a transient increase appearing at day 12. These results are based on a measure of fucose incorporation rate /mg protein and may be an indication of the proportional increase of fucosylated glycoproteins with age; thus, one may expect to observe a concomitant decrease in critical enzyme activity. There is some controversy in the literature as to the way in which developmental enzyme activities should be expressed. Some authors express activity as a function of protein content, giving specific activity values, while others favour the calculation of total enzyme activity, calculated on total wet-weight of the tissue. In the former calculation, the total amount of protein is not accounted for, while in the latter case, changes in wet-weight may be due to differential increases in cellular components; both calculations may therefore lead to distorted results. Probably the most accurate way in which to express enzyme

activities is in terms of cell DNA content, as this is presumed to remain constant during development. My results (calculated in terms of mg protein for comparative purposes) indicate that chick fucokinase activity increases linearly until day 20 and thereafter levels off. Leon et al (1982), in an investigation on the developmental profile of rat fucokinase activity, found that there was no change in activity over the 35 day post-natal period. However these workers found that fucosyl-transferase activity (the next enzyme in the pathway) increased from a specific activity level of 2 at 3 days to one of 9 at post-natal day 20. This increase in activity is thought to be associated with a period of increased myelination. Unfortunately, no such literature is available for the chick brain. Indeed, although there is a large body of literature available on the development of the embryonic chick brain (Rogers 1960), there is little published information on post-hatch development. Dolezelova et al (1974), in a study of the developmental profile of specific transmitter enzyme activities showed that changes in activity may be correlated with specific neuronal processes. Analysis of choline acetyl-transferase (ChAT), monoamine oxidase (MAO) and dopamine-B- hydroxylase (DBH) suggested that changes in activity could be associated with 'a rapid new phase of maturation and biochemical activity 2 - 4 days post-hatch, involving the functional relationship of ganglion cell bodies to their terminals, due to the development of visceral function's (Dolezelova et al 1974). They also suggested that at 7 days post-hatch, maturation of synapses and neuronal development is complete in the chick brain; this coincides with maximal activity of ChAt , MAO and DBH. My results show that fucokinase activity increases up until day 20 and may indicate that fucose-containing glycoproteins are not specifically and intrinsically concerned with developmental and maturational processes. There are no data available

on the developmental profiles of the other enzymes involved in the fucosylation of glycoproteins. As brain fucose is largely associated with membrane glycoproteins, perhaps the observed increase in fucokinase activity is indicative of the turnover rate, or total concentration of fucose-containing glycoproteins in the brain, and reflects changes in membrane composition, leading to membrane remodelling and plasticity.

The data from the diurnal study of fucokinase activity (Figure 3.4), indicate that there is a significant difference in activity, ($F=4.54$, df 11,64, one-way ANOVA), throughout a 24 hour period, with peaks in activity occurring at 11.00 hr and 07.00 hr. On separate analysis of diurnal activity in the two hatches, activity is not apparently influenced by a major biological cycle, the sleep/wake cycle (no effect was observed with different light conditions; see Figure 3.3A (constant light), and 3.3B (lights on and off at 08.00 and 20.00 respectively). Leon et al (1981), in a study of diurnal variation of rat brain fucosyltransferase, found that there was a 50% difference between maximum and minimum activity levels. These levels were not associated with any particular time of day, and were not synchronized to the light/dark cycle. Perhaps we can deduce from this that fucokinase activity (in both rat and chick) is not influenced by any external factors, in particular the extent of locomotor activity; this assumes that changes in locomotor activity coincide with the light/dark cycle, (which has not been investigated with the chick). If this is so, fucokinase activity may be controlled specifically by endogenous feed-back systems and substrate concentrations. Killer et al (1979) have shown that rat fucokinase activity is inhibited by GDP-L-fucose, which exerts feed-back inhibition (to prevent the accumulation of this substrate in this assay, a high concentration of potassium flouride (130 mM KF), which inhibits phosphorylation

reactions, was included in the incubation buffer; see Popov et al (1983). The most important observation from the diurnal study is that fucokinase activity does vary significantly throughout a 24 hr period, and therefore it is important that tissue for enzyme analysis is prepared at the same time of day for each experiment. This eliminates a possible confounding variable in analysing enzyme activity in chicks from different hatches.

In order to determine the physiological optima for fucokinase activity, with the aim of obtaining information on activation and inhibition mechanisms, a comprehensive study of the effect of a range of ions and nucleotides, was undertaken. Experiments with various Ca^{2+} concentrations indicated that high levels lead to inhibition of fucokinase activity. This result is difficult to interpret, as increasing levels of Ca^{2+} are invariably associated with neuronal activation mechanisms. If one is to consider the mechanisms associated with neuronal activation and transmitter release, an increase in intracellular Ca^{2+} (in the uM range) is an important, integral process. Calcium ions are involved in the two main facets of synaptic transmission; release of neurotransmitter from the pre-synaptic terminal and in the modulation of the post-synaptic response. In both processes, the actions of calcium ions are complex and probably involve cascades of molecular events, the nature of which are unknown at present (reviewed by Rodnight et al 1983). Also, the involvement of Ca^{2+} in a range of Ca^{2+}-dependent protein kinases and phosphorylation processes is well documented (Hock & Wilson 1984). In particular, increases in intracellular calcium associated with the physiological processes of training are documented in the literature, as discussed in Chapter 1.

As fucokinase is a cytoplasmic enzyme, it is exposed to varying concentrations of intracellular Ca^{2+}, ranging from high levels (1 uM) due to neuronal activation and transmitter release, to resting-state levels in the range of 0.1 um - 0.01 um, and perhaps lower levels if intracellular Ca^{2+} is sequestered into mitochondria or the endoplasmic reticulum (Somlyo 1984). Another important way in which intracellular Ca^{2+} levels are reduced is by the activation of calmodulin, forming calmodulin-Ca^{2+} complexes which may be associated with fucokinase activation. In a study of the properties of endogenous protein phosphorylation in chick and rat synaptic membranes, Sorensen & Babitch (1983) found that chick membrane proteins were activated to a greater extent by Ca^{2+}-calmodulin than by C'AMP. That inhibition of fucokinase activity in vitro was elicited by increasing calcium concentrations, the recorded Ic_{50} being 50 uM, would indicate that this is not a likely physiological mechanism in vivo, as the maximum physiological intracellular calcium concentration is 5 uM (Somlyo 1984).

Studies on magnesium revealed that a 3.3 mM concentration gave maximum activity, which is higher than that reported for physiological concentrations. Free magnesium concentration in brain tissue is considered to be 1mM. When manganese was substituted for magnesium, a maximum activity of 60% of control values was obtained with 0.5 mM. It would seem, therefore, that fucokinase requires a divalent cation for activity, Mg^{2+} being the most effective, or alternatively, that the mechanism of activation of both Mg^{2+} and Mn^{2+} may be associated with the selective inhibition of intracellular Ca^{2+} fluxes. Both Mg^{2+} and Mn^{2+}, Mn^{2+} more so than Mg^{2+} are capable of blocking stimulus- induced Ca^{2+} influx into pre-synaptic terminals (Natchshen & Blaustein 1980). These studies compare well with those of Richards & Serif (1977), in which the ionic

requirements of canine thyroid fucokinase were investigated. These workers also found that Mg^{2+} was the most effective cation in eliciting maximal activity, while 90% of control activity was obtained with Mn^{2+}. Other ions, including Ca^{2+}, were essentially ineffective.

In order to determine if fucokinase activity was affected by various concentrations of nucleotides (as possible protein kinase effector molecules), the effects of C'AMP and C'GMP were investigated. No increase in activity was observed with low concentrations while mM concentrations led to inhibition. If fucokinase activity is stimulated by C'AMP, then the inhibitory effect observed with high concentrations, may indicate that inhibition is elicited via a negative feed-back system, through inhibition of adenylate cyclase. This would only apply if there was contamination in the isolated soluble fraction of membrane-bound cyclic adenosine. Alternatively, C'AMP may act by inhibiting the action of KF, thus allowing the next enzymatic sequence to occur; the formation of GDP-fucose from fucose-1- phosphate via the soluble enzyme fucose pyrophosphorylase. This process would then reduce the amount of fucose-1-phosphate obtained. However, the concentration of nucleotides used in this study, which led to inhibition of fucokinase activity were higher than physiological concentrations. These results are contrary to those reported by Richards et al (1978), in which C'GMP had a stimulatory effect on thyroid fucokinase while all other nucleotide mono-, di-, and triphosphates (except ATP) were ineffective. A number of guanosine nucleotide sugars were also found to increase activity, in particular GDP- -D-mannose (Richards et al 1978), but it is assumed that activation is triggered by the specific guanosine portion of the molecule rather than the attached sugar molecule. It seems however, that metabolite control of chick fucokinase is not regulated by C'AMP

or C'GMP.

After passive avoidance training increases of 15%, and 20% in fucokinase activity were found one hour and six hours in (M) trained over (W) control chicks. No increase was found at the other train-test intervals. The absolute values of fucokinase activity recorded 1 hour after training correspond well with those obtained by Lossner & Rose (1983), who conducted a similar study. As was mentioned previously, increased fucose incorporation as a result of training chicks persisted for up to twenty-four hours (Burgoyne & Rose 1980a; Sukumar et al 1980). Perhaps this phenomenon is related to increased fucokinase activity at least until six hours after training, followed by activation of fucosyl-transferase. The effects of PAL training on chick fucosyl-transferase has not been investigated, but, using other paradigms where increases in fucose incorporation have been studied (Burgoyne & Rose 1980b; Jork et al 1982), no change in fucosyl- transferase has been documented.

In this investigation, an increase in fucokinase activity was initially localized in the left forebrain base one hour after training and consequently in the right roof after six hours. Lossner & Rose (1983), in a similar study, reported a significant change in activity (14%) in the right base, and a non-significant increase (11.3%) in the left base one hour after training. I cannot explain this inability to reproduce results showing an increase in a specific hemisphere, but I think one can be confident that there is a significant increase in activity in at least one region of the forebrain base, one hour after training. One can make several suggestions concerning the difference in these results, but probably one of the major factors is the intrinsic variability in the behaviour of a group of chicks (referred to in Chapter 2). The involvement of the forebrain base in the

cell-biology of training has been well documented. This region contains two specific nuclei, the lobus parolofactorius (LPO) and the paleostriatum augmentatum (P.A.), and both have been cited as areas of increased metabolic activity as a result of training, as investigated by 2-deoxyglucose (2-DG) techniques (Kossut & Rose 1984). A more recent study by Rose & Csillag (1985) has shown that there is an increased uptake of 2-DG in the left LPO of (M) trained over (W) control chicks, when 2-DG is injected five minutes before or ten minutes after training. Furthermore, the forebrain base has also been the area in which an increased fucose incorporation has been detected, one to four hours after training (Rose & Harding 1984). At the train-test interval of six hours, the significant increase in fucokinase activity was located in the right forebrain roof, which contains the hyperstriatum ventrale. In the experiments of Rose & Csillag (1985), an increased uptake of 2-DG was found in the right MHV of trained chicks when the label was injected ten minutes after training. Consequent to imprinting, biochemical changes have also been located in this region (IMHV) (Rose 1977; Horn 1981). It is interesting that the increase in activity shifts from the base to the roof, and this may indicate that the LPO and PA are critical for initial fucokinase activation, with subsequent increase in fucose incorporation, and that the hyperstriatum ventrale in the roof then becomes important in the process of information storage. The involvement of one region and the subsequent shift to a second region resembles the hypothesis of engram formation proposed by Horn (1981) in a study of imprinting in chicks (discussed in detail in Chapter 2). He suggested that the left IMHV is the initial critical site of information storage (S), and that later, the right IMHV becomes involved in the consolidation of the store ('S').

In conclusion, this set of experiments shows the developmental and diurnal profiles of chick fucokinase activity. Activity is reduced by the addition of calcium, and the presence of a divalent cation, magnesium being the most effective, is necessary for maximal activity. After training chicks on a passive avoidance paradigm, significant increases in fucokinase activity were found one hour and six hours after training. These increases were lateralized to the right base and left roof, respectively.

The aims of the following experiments were to identify the specific glycoproteins in which the observed increase in fucose incorporation in vivo was detected. I also wanted to investigate further the mechanisms by which this increase in incorporation due to training occurred.

CHAPTER 4

FUCOSE INCORPORATION IN CHICK FOREBRAIN SLICES

Having established that there may be a relationship between increased fucokinase activity and an increased fucose incorporation in chick forebrain *in vivo* as a result of training, I attempted to replicate this increase *in vitro*. My initial aims were to establish if an active fucose incorporation system could be achieved in chick forebrain slices, and then to investigate if the effects observed after training, *in vivo* could be replicated *in vitro*. The advantages of an *in vitro* system include a greater range and specificity of experimentation, and with this property, the ultimate aim was to isolate and identify the glycoproteins into which the increase in fucose incorporation was observed. Such identification of specific glycoproteins associated with the training procedure is more difficult *in vivo*, as the relative amount of label incorporated into proteins is very low.

Researchers have used *in vitro* techniques for many years as they offer a range of advantages, in particular greater accessibility and a greater opportunity to manipulate the factors affecting normal physiological function. Many of the studies employing *in vitro* techniques have been concerned with the analysis of protein metabolism in brain tissue, utilising labelled amino-acids. Tissue that has been

used includes slices (Folbergrova 1966), cell-free homogenates (Peterson & McKean 1969) and chopped tissue homogenates (Austin & Morgan 1967). Using in vitro techniques, the monitoring label can be applied directly to the tissue in question and a good recovery rate of label can be obtained such that the efficiency of the system is maximal. In in vivo work, after intracranial or intraperitoneal injection of label, a high proportion of it does not reach the pertinent area. This may be due to differential uptake into different cellular compartments, or differences in the kinetics of uptake and incorporation of precursor arising from the particular route of application. Also with in vivo studies there are immense problems associated with assessing intracellular pool-sizes of the precursor being used, leading to problems in interpreting results (reviewed by Lajtha & Piccola 1971).

With the development of optimal conditions for maintenance of brain tissue in vitro, a number of problems were eliminated, in particular, the quantity and thus the cost of radioactive label necessary for each study was greatly reduced. However, in vitro systems are not without limitations; one great problem concerns the maintenance of maximum viability of the isolated tissue. The tissue must initially be removed from the organism, and during this process, inevitable mechanical and osmotic damage will be incurred. Normally it is then bathed in a physiological medium (an environment closely resembling that in the intact organism), such that essential metabolic processes can take place. As the exchange of metabolic compounds must occur via diffusion processes in the isolated tissue, this severely limits the size of the sample one can work with. Oxygen is probably the most important requirement and for adequate oxygenation, tissue should not be more than 0.4 mm in thickness (Field 1948). This limitation in thickness implies that in preparing tissue from many

parts of the mammalian nervous system for metabolic studies, two distinct processes are involved; (1) removal from the body and (2) slicing or chopping to yield the necessary, thin sections (reviewed by McIlwain 1966). With these limitations, a primary concern in experiments using isolated tissue including brain slices, has been to approximate more closely to the conditions of the *in situ* tissue. The advantages of using tissue from young animals as opposed to adults have been extensively studied by Dunlop *et al* (1977). This study involved comparing the developmental protein synthesis rates in rat brain, under both *in vivo* and *in vitro* conditions. It was found that the incorporation rates of labelled amino-acids in the young brain *in vitro* closely resembled those *in vivo*; rates of 70%-80% were recorded. However, in slices from the adult, only 20% of the *in vivo* incorporation rate was recorded *in vitro*. This may be due to the presence of active cell division in the developing brain, such that there is major recovery of the cell damage consequent to tissue isolation; such recovery may not possible in the adult *in vitro* preparation (Lajtha & Dunlop 1973).

The brain slice preparation is a most attractive *in vitro* system in that the cellular interconnections and structural elements of the tissue are relatively undamaged and the environment can be closely controlled during incubation in a physiological medium. Slices from rat brain tissue have been extensively used in *in vitro* studies of neurochemical function (Dunlop *et al* 1975; Jones & McIlwain 1971). Only one recent study, to my knowledge, has used chick forebrain slices in which an active protein synthesis system was established (Schliebs & Rose 1985), although Liu *et al* (1975) have described an active *in vitro* protein synthesizing system in embryonic chick brain tissue, specifically the post-mitochondrial supernatant portion from the cerebrum, cerebellum and optic lobes.

Most of the interest to date has concentrated on protein synthesizing systems *in vitro* in mammalian tissues, with limited study on chick tissue, and in particular, glycoprotein metabolism *in vitro*. I therefore decided to determine whether a stable and active glycoprotein synthesising system could be established in chick forebrain slices *in vitro*, using labelled fucose as a marker.

4.1 MATERIALS AND METHODS

Dissection of the chick brain was carried out (as previously described in Chapter 3), into forebrain roof and base regions with the aid of an araldite mould, and the hemispheres were separated. Each region was placed on ice and transferred to precooled filter paper at 4 C. Each region was then sliced to a thickness of 0.4mm using a McIlwain tissue chopper, and subsequently placed in pregassed (95% O_2, 5% CO_2), preheated (42 C) incubation medium. Individual slices were then separated and placed in prepared incubation vials for experimentation. Separating the slices took approximately 20 minutes, and served as the pre-incubation period.

Incubation

The incubation medium was a Hepes (N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid) buffer, based on that used by Dunlop et al (1977), pH 7.4, and was composed of final concentrations in mM: NaCL 118, KCL 7.4, $MgCl_2$ 1.3, $CaCl_2$ 2.6, K_2HPO_4 1.5, D-glucose 12 and Hepes 25. 6 ml of buffer was placed in each incubation vial, gassed for ten minutes and heated in a water-bath normally at 42 C, for a period of 20 minutes before the addition of the slices. After pre-incubation, 8-15 slices (3-4 mg protein) of forebrain tissue from each region were placed in separate vials. 100 ul of a 6mM fucose solution containing 3.7 KBq/ml U-L-^{14}C fucose (specific radioactivity 2.22 GBq/mmol, Amersham International) was added to each flask, which were securely capped. The flasks were then vigorously shaken in a water-bath at 42 C for up to 3 hours. After the incubation period, an aliquot of 200 ul of the medium was sampled to determine medium specific radioactivity, and 1ml

was retained for evidence of glycoprotein leaching from the slices. The reaction was then stopped by the addition of 6ml ice-cold medium and the flasks placed on ice. The slices were drained and washed twice with 6 ml ice-cold 10mM fucose to eliminate nonspecific fucose binding. Triplicate samples from each incubation were made by taking 5 slices (1 mg prot) from each flask, placing them in 2 ml ice-cold 10 mM fucose and homogenizing using a Polytron (setting 4) for 10 seconds. 200 ul of homogenate was removed for protein estimation (Lowry et al 1951). To the remaining 1.8 ml, 5 ml of an ice-cold 10% trichloroacetic acid (T.C.A.) solution containing 10 mM fucose was added, left for 10 min at 4 C and centrifuged at 3,000g for 10 min. The supernatants were retained for estimation of acid insoluble radioactivity. The pellets were washed twice with T.C.A. and finally dissolved in 2 ml protosol to which 10 ml of scintillation fluid (Cocktail T, BDH) was added. The 1ml aliquot retained from the external medium was treated in a similar manner. To reduce chemiluminesence, 1.1 ml water was added to each sample and left for 12 hours before counting in a Beckman LS.7500 Scintillation Counter at an efficiency of 80%.

Delipidation

In some experiments, the washed T.C.A. pellet was defatted to determine the proportion of fucose incorporation into glycolipids. The pellet was resuspended in 1 ml ethanol and centrifuged at 10,000g for 10 min. The supernatant was retained and the pellet washed in 1 ml of a chloroform:methanol (2:1) solution and centrifuged at 20, 000 g for 10 min. Finally, the pellet was washed again in 1 ml of ethanol. The supernatants from all centrifugations were combined and analysed for radioactivity.

Fucose incorporation into forebrain slices after training on P.A.L.

Chicks were trained on the passive avoidance learning paradigm (P.A.L.) as described in Chapter 2, and tissue slices incubated as above. Slices from the four dissected regions (L.R.,R.R., L.B. and R.B.) of 10 (M) trained and 10 (W) control chicks were assayed for fucose incorporation in the T.C.A. precipitable fraction.

Subcellular Fractionation

To determine the distribution of labelled fucose in subcellular fractions following training, slices from the right forebrain base of 6 (M) trained and 6 (W) trained chicks were homogenized in 2 ml of 0.32 M sucrose solution containing 10 mM fucose, and centrifuged at 1,000 g for 5 min. The pellet was resuspended and washed once, the supernatants (S1) were combined and the nuclear pellet (P1) was retained. (S1) was then centrifuged at 13,000 g for 20 min. The pellet, a mitochondrial, myelin and synaptosomal fraction (P2), was washed once and retained and the supernatants (S2) combined. Finally, (S2) was centrifuged at 80,000 g for 75 min giving (S3), a soluble fraction and (P3), an internal membrane (microsomal) fraction. For analysis, all pellets were resuspended in 500 ul of 0.32 M sucrose solution containing 10 mM fucose and an aliquot of 100 ul taken from each, added to 5 ml of T.C.A. and centrifuged. The resulting T.C.A. pellet was treated as above and radioactivity determined. Aliquots of 200 ul of the supernatants were treated in the same manner.

Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (S.D.S. PAGE)

In order to identify the glycoproteins into which fucose was incorporated as a result of training, tissue slices from the right base of 3 (M) trained and 3 (W) control birds were incubated separately in medium containing 370 Bq/ml L-^{14}C-fucose (a specific radioactivity 100 fold higher than that normally used). For this experiment, the medium volume was reduced to 2 ml. After incubation, the tissue was fractionated as above and the P3 fraction retained. Protein determination was by the method of Bradford et al (1976). 100 ug or 400 ug protein was washed with 1 ml 10% T.C.A. and left on ice for 10 min. It was then centrifuged at 3,000 g for 10 min and the precipitate retained. To remove all traces of acid, the pellet was extensively washed with 1 ml diethyl-ether:ethanol (1:1), and subsequently resuspended by sonication in a Soniprep 150 for 10 seconds in a 2.5% S.D.S. dissociation buffer (pH 6.8) for gel analysis. A gradient gel, 5% - 15% acrylamide with a 5% stacking gel, was prepared by the method of Laemmli et al (1970). Samples of the P3 fraction (100 ug or 400 ug protein) were then heated to 100 C for 5 min and placed on the gel. Samples of molecular weight markers were also run on the gel for identification purposes, and 2 ul of bromophenol blue was added to each sample as a tracking dye. The samples were then electrophoresed with a constant current of 30 mA for 7 hours. The gel was subsequently removed and stained overnight with Coomasie blue in a shaking water-bath. It was then destained with successive washings of a solution containing 10% acetic acid and 12.5% propanol. Each track on the gel was then scanned densitometrically with a Chromoscan 3 (Joyce Loebl) and photographed. Subsequently, each track was cut from the gel and cooled with dry ice before cutting

into 2mm slices with a McIlwain tissue chopper. The slices were then placed in glass vials and left overnight to hydrate (to remove all traces of the destaining solution), rehydrated by adding 500 ul of water to each and left for one hour. The excess water was removed and 8 ml econofluor (New England Nuclear) containing 5% protosol added to each vial. To aid the complete solubility of the gel, the vials were placed in a hot oven at 50 C for 30 min, and then allowed to cool. 24 hours later, radioactivity was determined using a Beckmann LS 7500 counter to 80% efficiency.

Electron Microscopy

In order to determine the integrity and state of preservation of the tissue, the ultrastructure of slices was analyzed before and after incubation. Slices prepared as indicated above were taken and either fixed immediately by immersion in 3% glutaraldehyde and 0.5% paraformaldehyde in 0.1 mM cacodylate buffer pH 7.4 (containing 3% sucrose), or fixed after a 3 hour incubation period. Following primary fixation for 60 min, the tissue was washed twice in 0.1 mM cacodylate buffer, post-fixed in 1% osmium tetroxide, dehydrated in an ethanol series and embedded in Spurr resin. Sections of silver-grey interference colour (approx 50 nm) were cut on a Reichert ultra-microtome OMV4, in a direction perpendicular to the plane of the original surface of the slice. Sections were then collected on 20 mesh copper grids and stained with lead and uranium salts before screening using a JO-EL 100 electron-microscope operated at 60 KV. Photographs were taken of the edges and internal structures of pre- and post-incubated tissue slices at instrument magnifications ranging from 3,000 to 10,000.

The ultrastructure of the isolated P3 fraction (microsomal fraction) obtained on subcellular fractionation of tissue slices was also prepared and analysed by electron microscopy. After isolation the tissue was fixed as above and 60 nM sections cut. Photographs were then taken at instrument magnifications ranging from 15,000 to 30,000.

Effect of Neurotransmitters and Calcium-free medium

The effect of a range of neurotransmitters on fucose incorporation was studied. Slices were prepared and incubated as indicated above. 100 ul of a range of transmitters of various final concentrations were placed into the medium; noradrenaline (NA) (10 mM-1 uM), dopamine (DA) (10 mM-0.05 mM) and 5-hydroxy- tryptamine (5-HT) (0.1 mM-1 uM). With these transmitters, the medium was supplemented with 100 uM tropolone and 100 uM pargyline to inhibit catechol-o-methyl-transferase and monoamine oxidase activity, respectively; 100 uM ascorbic acid was added as an antioxidant. Various concentrations of acetylcholine (ACh) (10 mM-0.01 mM) were also tested , and 100 uM eserine was included in the incubation medium to inhibit acetylcholine esterase activity. Each range of transmitter concentrations was assayed with a control preparation (no transmitter added), and each transmitter was studied in three separate experiments.

In another set of experiments, slices were incubated in a calcium-free buffer (treated) and fucose incorporation compared to that found in slices incubated in the normal medium, which contained 2.6 mM calcium (control).

After the standard incubation time of 3 hours, the slices were

washed extensively as described above and fucose incorporation in the T.C.A. insoluble fraction determined in control and treated slices.

Calculations -

Fucose incorporation was calculated as nmoles/gprot/hr, based on the specific radioactivity of fucose in the incubation medium in each flask. The contribution of free fucose in each slice to the overall fucose concentration was assumed to be negligible, as discussed in Chapter 2. In order to eliminate variations in the behavioural experiments due to hatch differences, the final data were standardised by normalising around the grand mean. This standardization procedure was also used in the calculation of fucokinase activities in Chapter 3. This was justified by the experimental protocol, which involved a balanced design of (M) trained and (W) control chicks and brain regions. Statistical comparisons were made using a two-tailed, unpaired Student's t-test. A one-tailed, unpaired Student's t-test was used on data when a specific prediction concerning the direction of a difference was tested.

4.2 RESULTS

Characteristics of Incorporation of U-L-^{14}C fucose into chick forebrain slices

Initial experiments were carried out to determine the optimal conditions for maximal fucose incorporation into a T.C.A. insoluble fraction. Figure 4.1 is a graph of the temperature dependence of fucose incorporation over a 3 hr period. Incorporation increases with temperature up to 42 C, with a maximum recorded incorporation of 37 nmoles/g prot/hr. Thereafter, incorporation was reduced at higher temperatures. Slices were incubated for various times ranging from 0 - ^{3}hr. Figure 4.2 shows that incorporation into the T.C.A. insoluble portion was linear with time until 3 hr, while incubation times longer than this led to significant loss of the medium by evaporation (with consequent distortion of results). To determine the time of incubation at which the concentration of intracellular fucose equilibrated with that of the external medium, radioactivity in the T.C.A. insoluble fraction was analysed at various intervals after incubation. Figure 4.3 shows that fucose incorporation into the acid soluble pool was linear until 60 min, and subsequently levelled off at a concentration of some 70% of that in the external medium (Table 4.2), assuming a tissue density of 1.0 and a protein:wet weight ratio of 10%. Figure 4.4 shows that over a 3 hr incubation period at 42 C, incorporation was dependent on medium fucose concentration up to a saturating concentration of 0.05 mM.

On the basis of these preliminary experiments, the following conditions were chosen for subsequent assays: a temperature of 42 C for a 3 hr incubation period, and a final fucose concentration of 0.1 mM with specific radioactivity 3.7 KBq/ml.

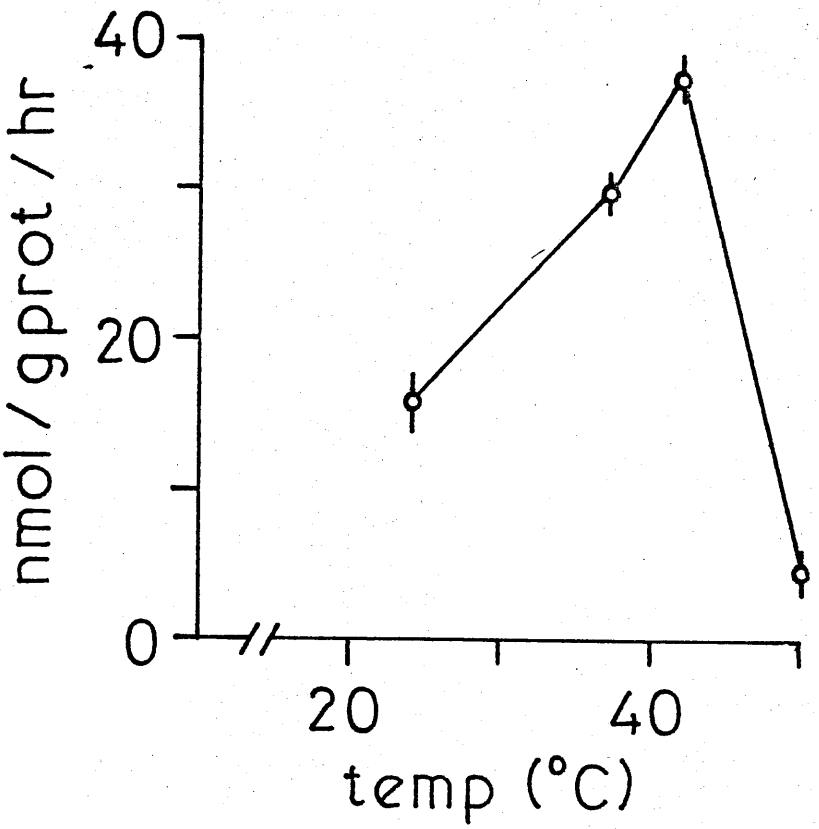


Figure 4.1. Chick forebrain slices were incubated as described in Materials and Methods, in a glucose-containing medium containing 3.7 KBq/ml of U-L ^{14}C fucose. The incorporation of fucose into an acid-insoluble fraction was determined under a range of incubation temperatures (25 to 50°C). Values represent mean ± SEM, where n = 3.

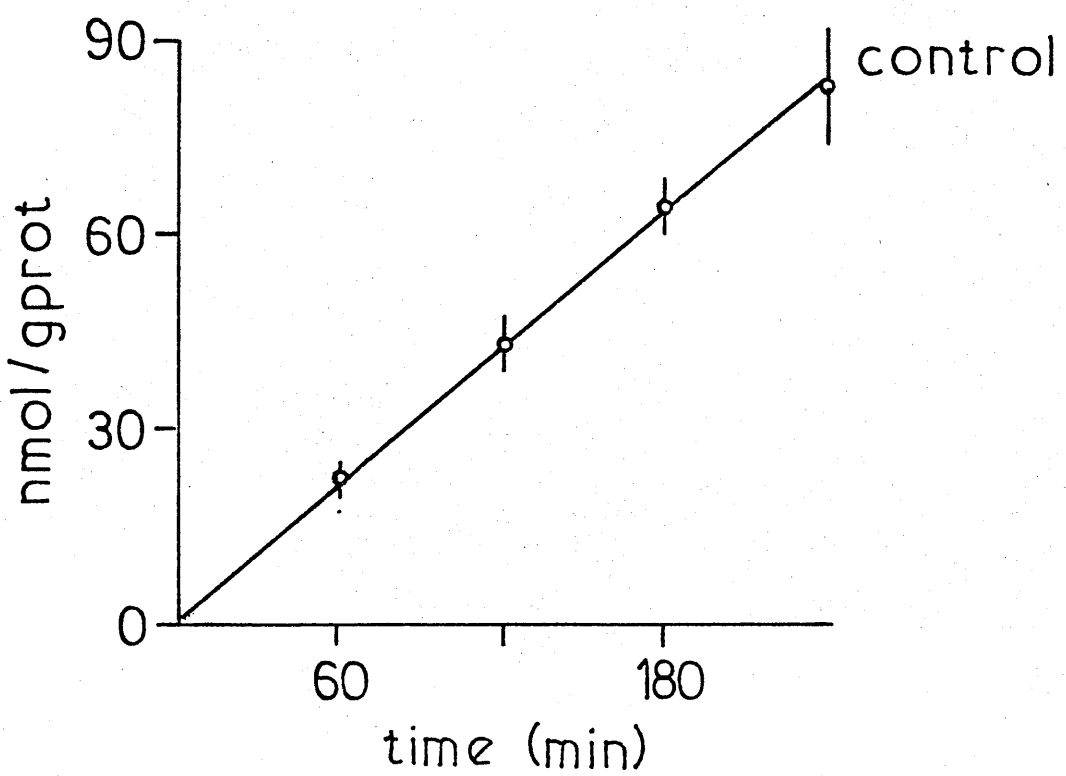


Figure 4.2. Slices of chick forebrain were prepared and incubated as described in Materials and Methods. At various intervals ranging from zero to 240 min, slices were removed and U-L ^{14}C fucose incorporation into an acid-insoluble fraction was determined. Values represent mean $\pm$ SEM of three separate determinations.

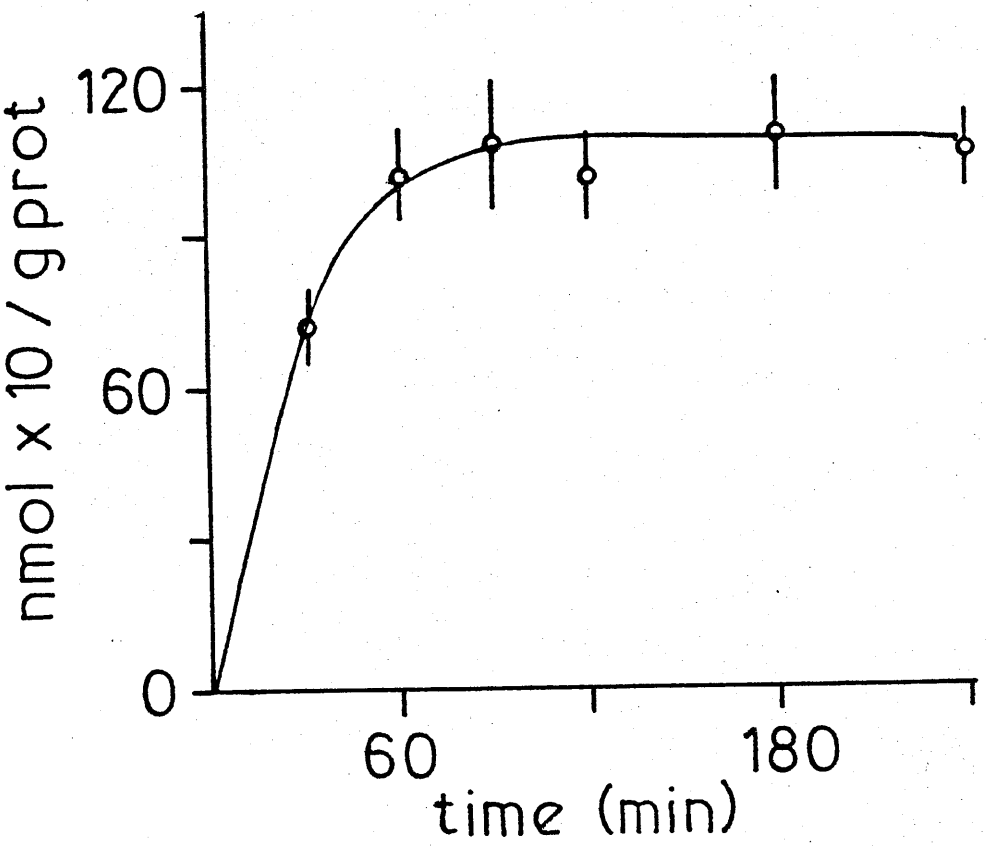


Figure 4.3. Slices of chick forebrain were prepared and incubated in a glucose medium containing ^{14}C fucose. At various times (zero to 240 min), slices were removed and fucose incorporation into the soluble pool was determined in the TCA soluble portion. Values represent mean $\pm$ SEM of three separate determinations.

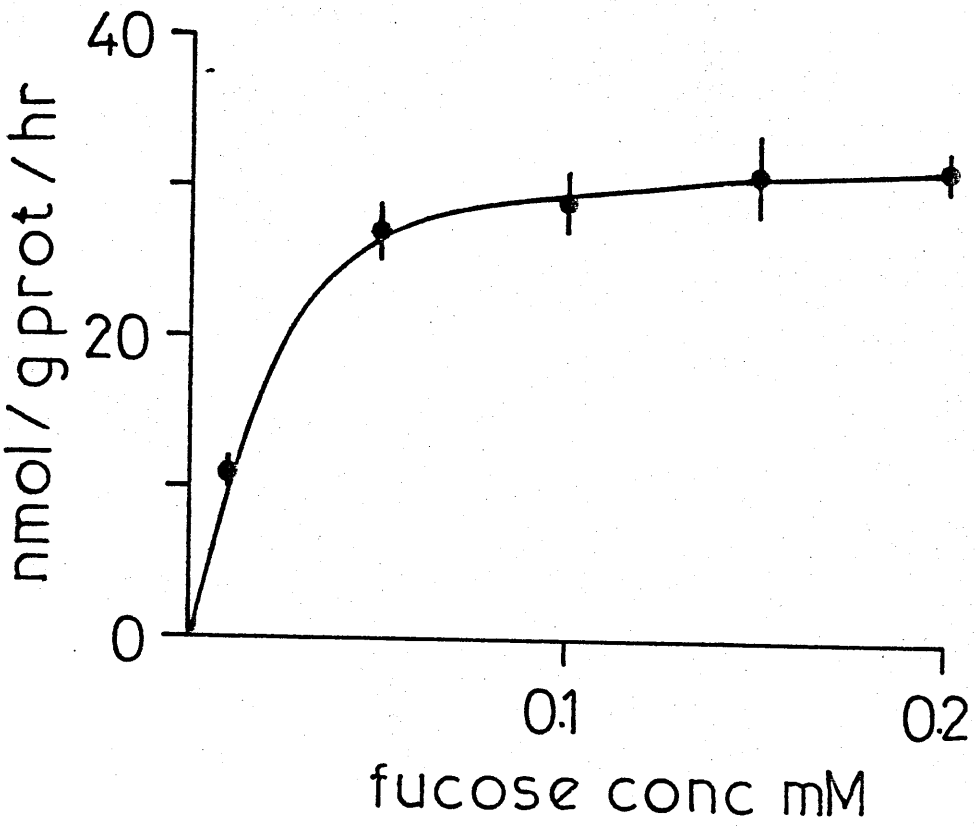


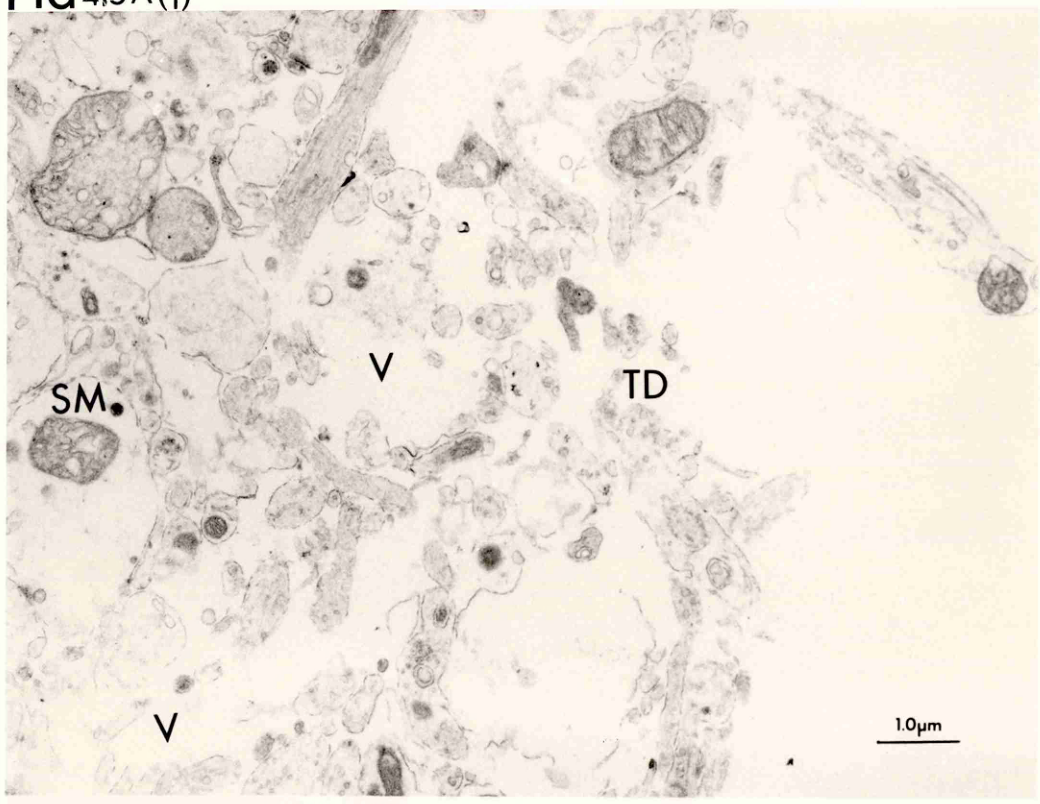
Figure 4.4. Slices of chick forebrain were prepared and incubated as described in Materials and Methods, in a medium containing a range of fucose concentrations (0.025 to 0.2 mM). The incorporation of fucose into an acid-insoluble fraction was determined after a period of incubation of 3 hr. Values represent mean $\pm$ SEM, where n = 3.

Ultrastructural analysis of slices before and after incubation

To determine the degree of tissue damage due to mechanical cutting with the McIlwain tissue chopper and the integrity of the tissue before and after incubation, an electron microscopic study was conducted. Figure 4.5A (1),(2), 4.5B (1),(2), are electron micrographs (magnification 18K) of the cut edge before and after incubation, respectively. It is evident that there is appreciable tissue damage (TD), with evidence of extensive membrane fragmentation and vesiculation. After incubation, vacuolation (V) and swelling of mitochondria (SM) are evident especially around the cut edges of the tissue.

Figure 4.6A (1),(2), 4.6B (1),(2), are electron micrographs (magnification 18K) of an area in the centre of tissue slices before and after incubation, respectively. The integrity of the tissue before incubation appears normal, with evidence of close compaction of cell organelles and evidence of normal cytoplasmic components; mitochondria (M), Golgi (G), pre- and post-synaptic densities (P), synaptic vesicles (S) and dendrites (D) can be seen. These cytoplasmic organelles are also evident in the post-incubated slice, but there is also some minor evidence of mitochondria swelling (SM) and vacuolation (V). However, the general state of the tissue shows little distortion.

FIG 4,5A (1)



(2)

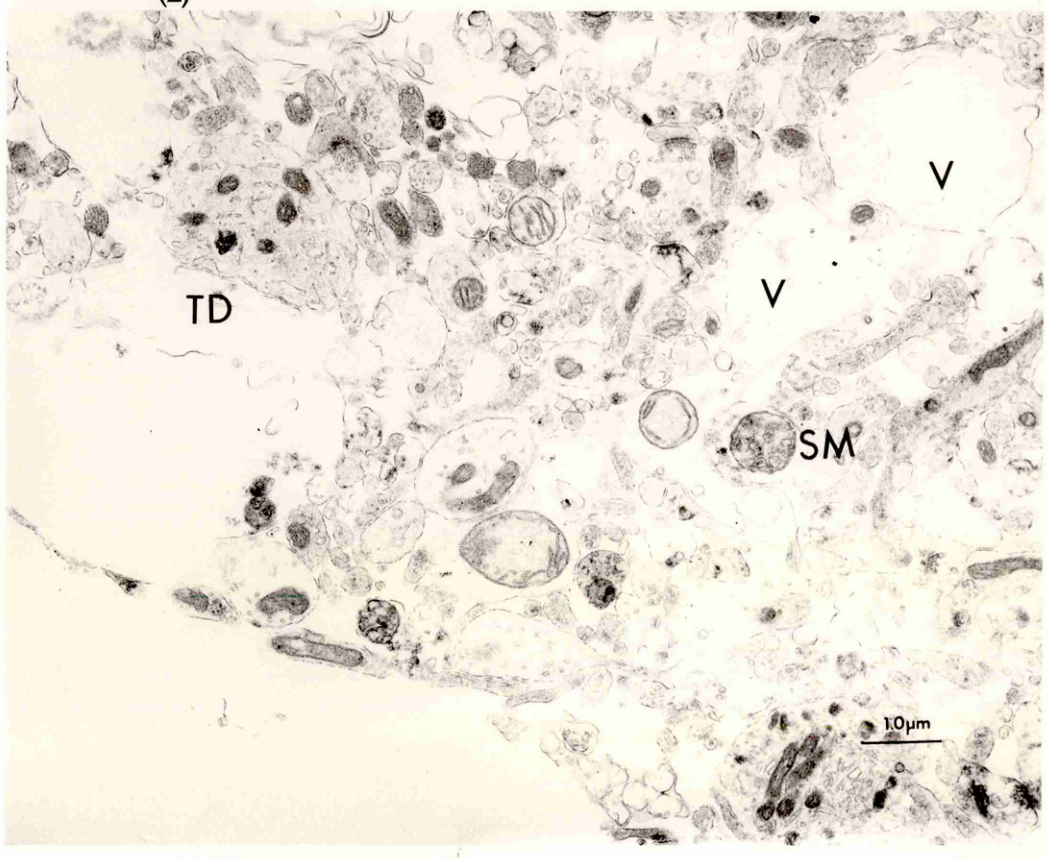
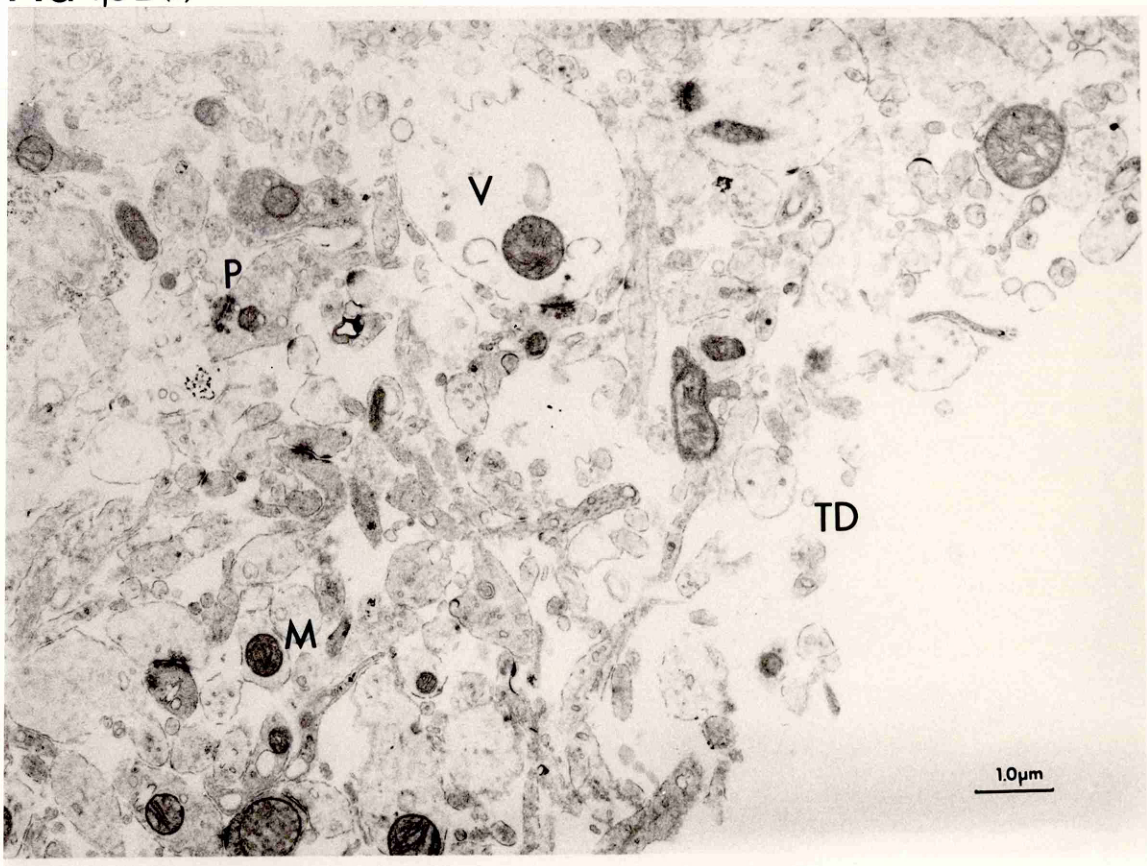


FIG 4,5 B (1)



(2)

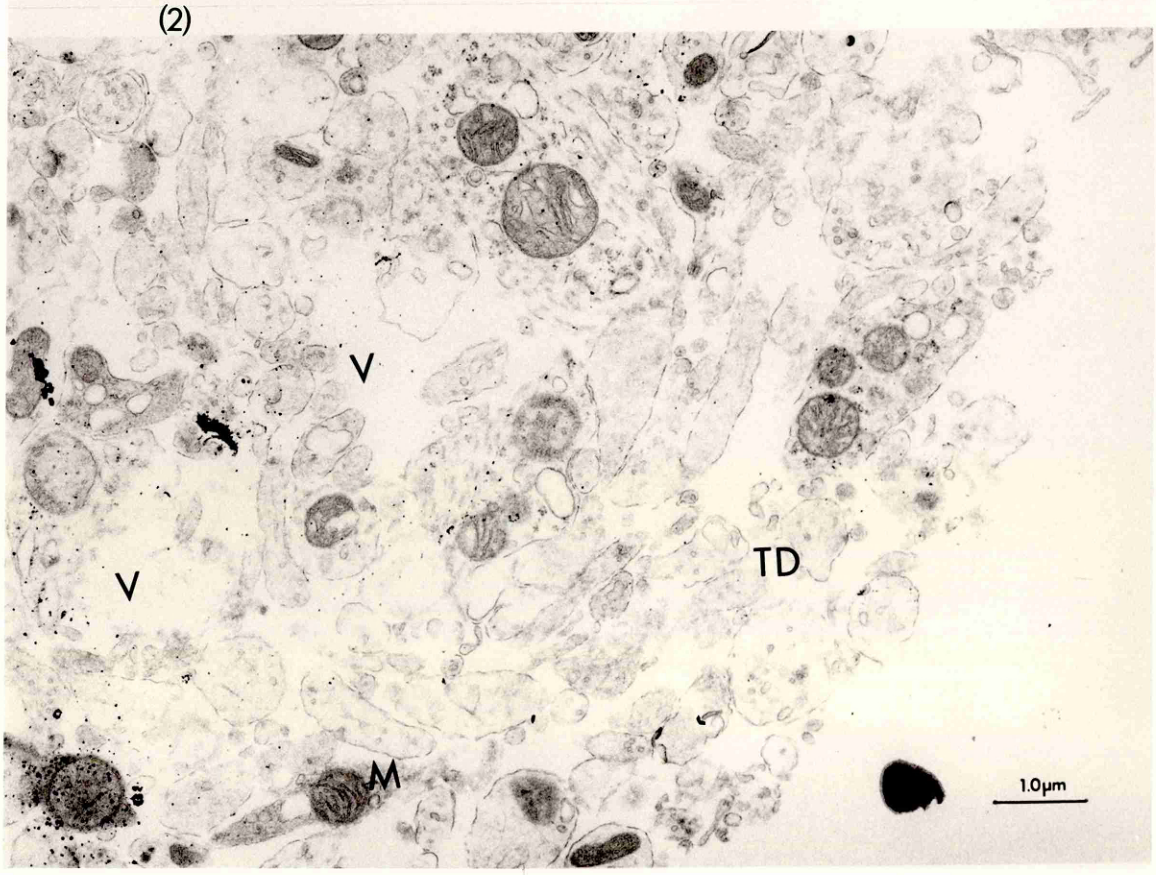
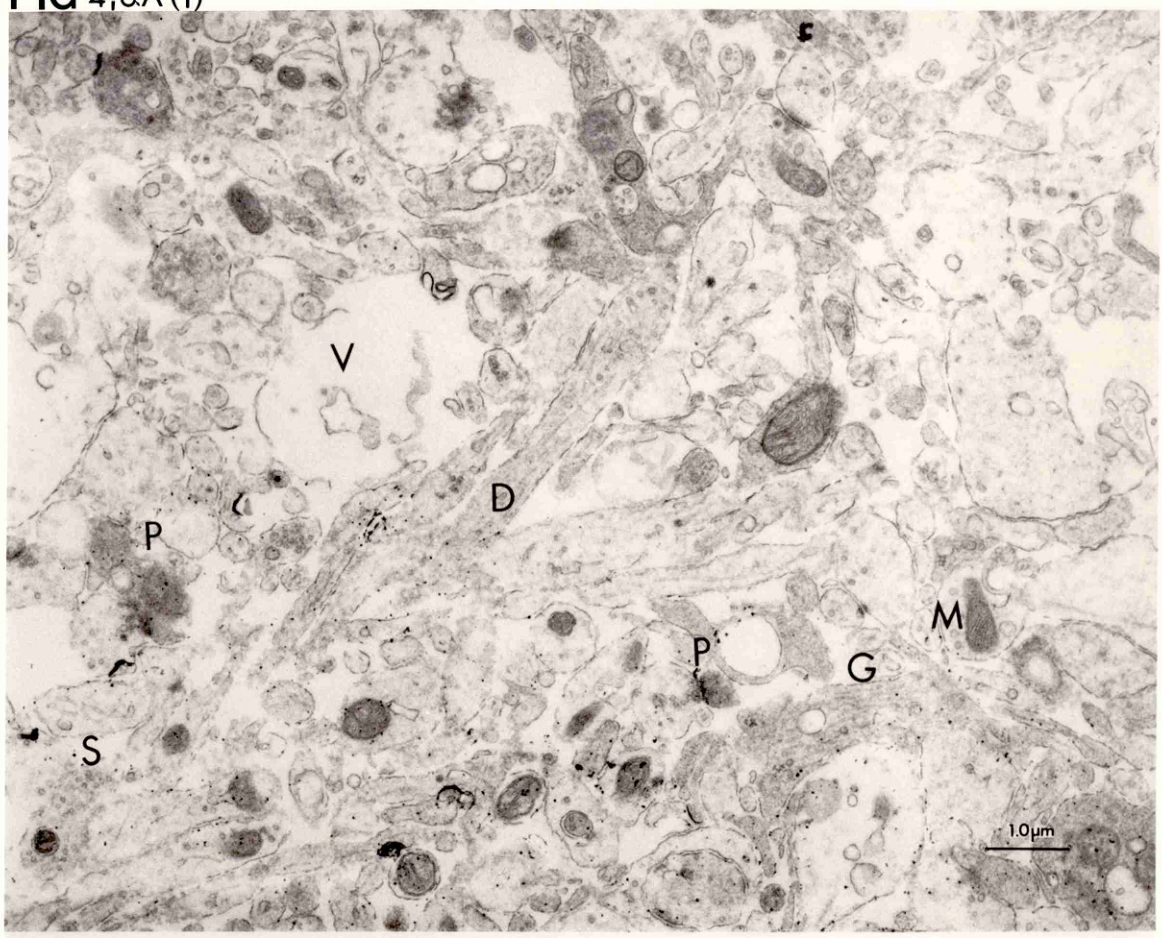


FIG 4,6A (1)



(2)

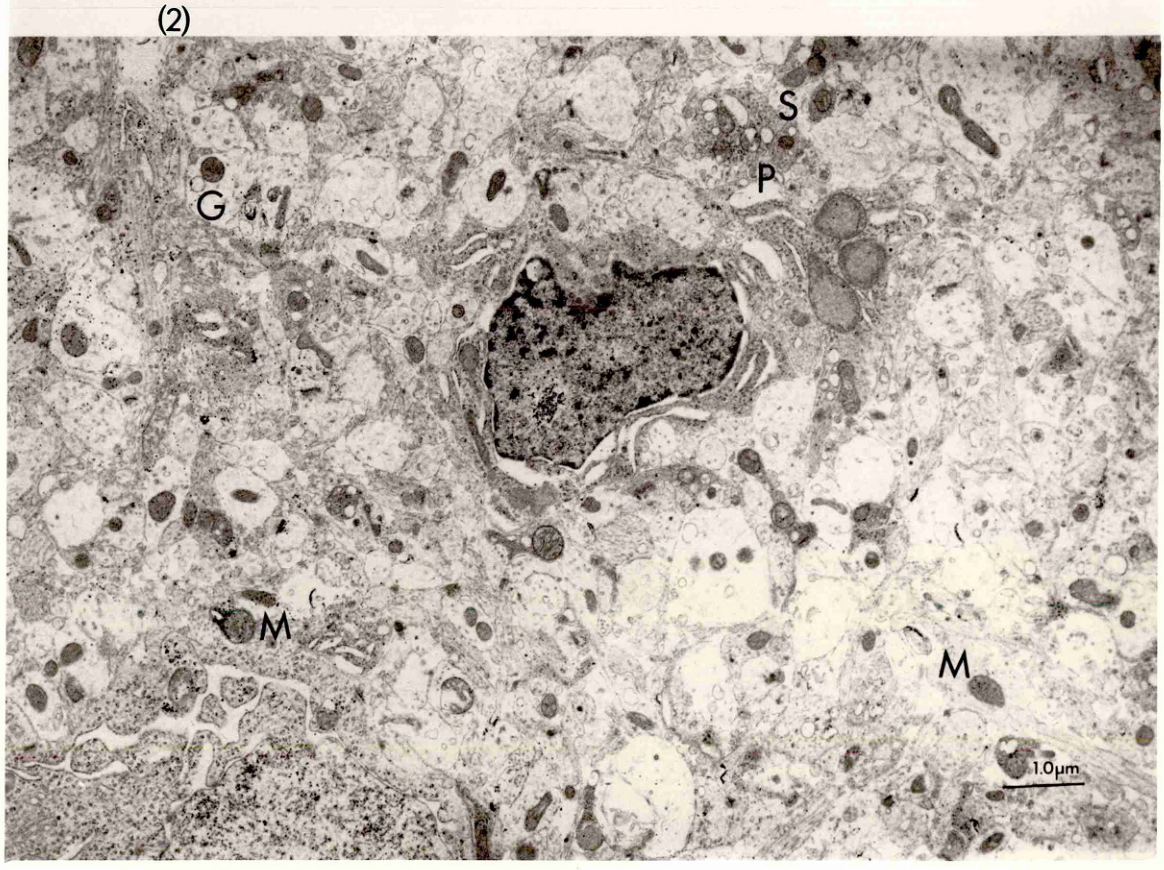
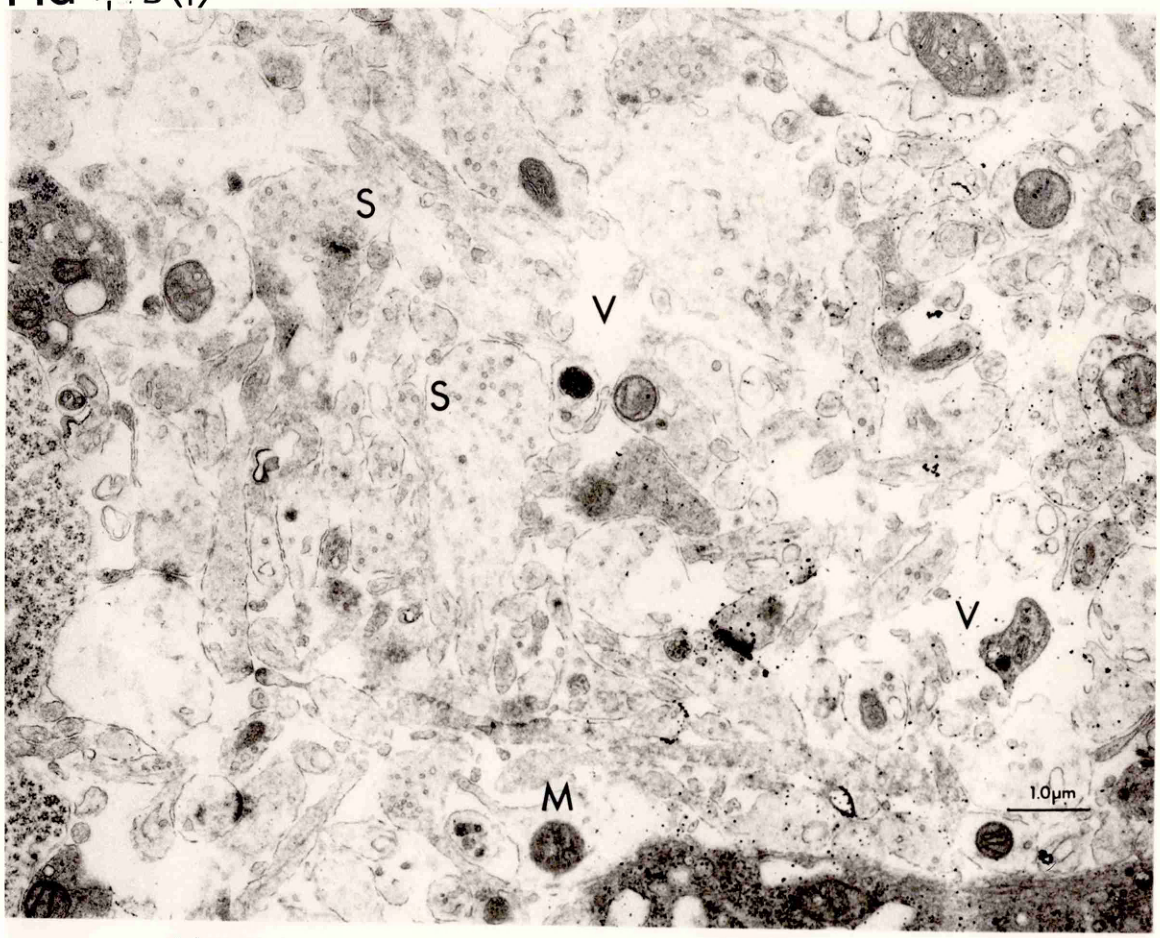
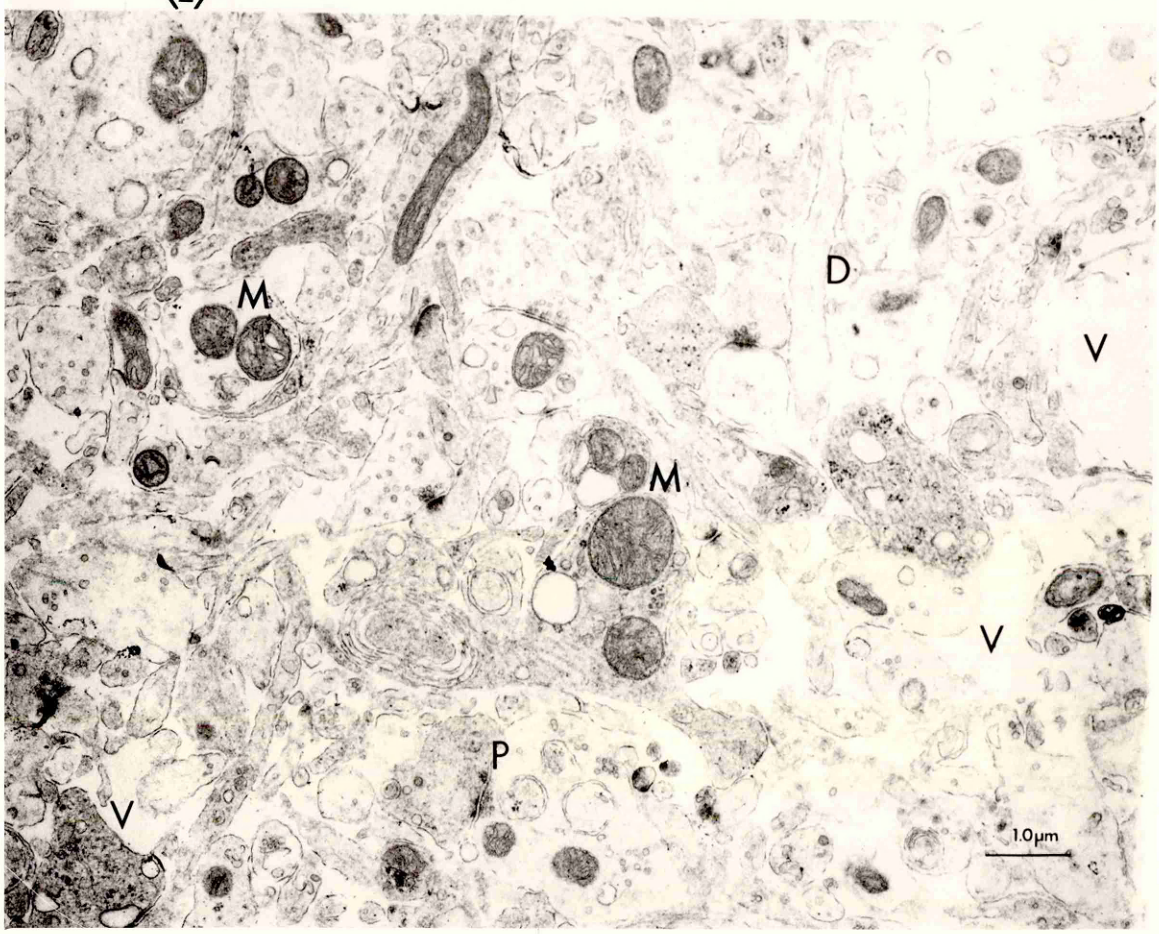


FIG 4,6 B〈1〉



〈2〉



Delipidation

On analysis of the ethanol:chloroform methanol extract of three washed T.C.A. pellets, a mean of 18% of the total radioactivity found in the T.C.A. insoluble fraction of homogenate was detected. This suggests that most of the labelled fucose was associated with glycoproteins and not glycolipids.

Table 4.0

Analysis of Fucose Incorporation into the Lipid Fraction.

	Total Counts Recovered		
Pellet	TCA Fraction	Lipid Extract	$\frac{\text{Lipid Extract}}{\text{TCA Fraction}} \times 100$
1	1276	207	16
2	981	205	21
3	1164	252	21

Values represent the analysis of three separate pellets.

Fucose incorporation into regions of the chick forebrain after training.

In vitro fucose incorporation was studied in four regions of the chick forebrain following training on a passive avoidance task. Slices were prepared from birds 30 min and 24 hr after training and incubated as previously described. As is seen from Table 4.1A, 4.1 B, fucose incorporation rates were generally higher in the roof regions than in the base regions. Table 4.2 shows that 30 min after training there was a significant difference in the ratio of radioactivity in the intracellular space:radioactivity in the external medium in the R.B., L.B. and the R.R. of trained chicks compared with controls. This indicates that there is a training-related increase in uptake of fucose in these regions. However, a significant increase of 16% ($P<0.01$) in fucose incorporation into the TCA insoluble fraction was observed only in the right base of trained chicks after 30 min compared to controls (Table 4.1A). No increase in fucose incorporation was observed in any region 24 hr after training (Table 4.1B). It should be noted that the data from Table 4.1A and 4.1B cannot be directly compared as they were two separate experiments and were statistically analysed separately.

To determine the fraction in which this increased fucose incorporation was taking place, a subcellular fractionation study of slices from the right forebrain base of 6 (M) trained and 6(W) control chicks was conducted. Table 4.3 shows that there is an unequal distribution of label in all fractions studied, with a large incorporation occurring in the P1 fraction (a nuclear fraction) and in P3, a microsomal fraction. However, the greatest incorporation of label was found in the S3 fraction, the soluble portion, which would suggest the presence of secreted fucose-containing glycoproteins.

This was an unexpected result, as fuco-glycoproteins are predominantly membrane bound: up to 80% in the rat (Popov et al 1976a). However, the increase in fucose incorporation, observed after training was found in the insoluble portion of the P3 fraction of the right forebrain of trained animals over controls. This increase, of 14.5%, was significant using a one-tailed t-test ($P<0.05$). This was legitimate as I had previously established that a significant increase in fucose incorporation did exist between the two groups, and I was further testing to find where it occurred. On analysis of fucose incorporation into the TCA insoluble portion of the external medium (Extern.Med.), to determine if there was any glycoprotein leaching from the slices, only 6% of the total radioactivity in the homogenate was found. There was no significant difference in fucose incorporation in the TCA insoluble portion of the Extern.Med. obtained from (M) trained and (W) slices.

Table 4.1

Fucose incorporation into slices from brain regions of (M) trained and (W) control chicks trained on a passive avoidance paradigm.

Region	M	W	M/W x 100	t
	nmol/ gm prot/ hr			
(a) 30 mins				
Right Base	36.69 ± 1.34	31.57 ± 1.31	116	2.923*
Left Base	35.26 ± 3.21	32.28 ± 1.51	109	1.676
Right Roof	38.45 ± 1.56	39.52 ± 0.87	97	0.560
Left Roof	38.18 ± 0.93	37.91 ± 1.38	101	0.163
(b) 24 hrs				
Right Base	28.59 ± 1.12	27.45 ± 1.47	104	0.167
Left Base	29.30 ± 4.95	26.21 ± 0.67	112	1.812
Right Roof	32.18 ± 0.72	31.32 ± 1.16	103	0.633
Left Roof	32.99 ± 0.92	32.83 ± 1.10	101	0.113

Chicks were trained as described and fucose incorporation into forebrain determined (a) 30 min (n = 11) and (b) 24 hr (n = 10) after training. Values are standardized means ± SEM for (a) and (b) separately.

* $P < 0.01$ (Student's t-test, two-tailed).

M: methylanthranilate, W: water.

Table 4.2

Ratio of specific radioactivity in the intracellular space to that in the external medium.

$$\text{Ratio} \frac{\text{Radioactivity intracellular space.}}{\text{Radioactivity external medium}}$$

Region	M	W	M/W x100	t
Right Base	0.868 ± 0.028	0.665 ± 0.025	118	5.346**
Left Base	0.808 ± 0.028	0.652 ± 0.019	124	5.160**
Right Roof	0.759 ± 0.024	0.652 ± 0.028	116	2.818*
Left Roof	0.717 ± 0.034	0.660 ± 0.017	109	1.488

Ratios were calculated from the experimental results in Table 4.1a. as:

$$\frac{\text{specific radioactivity in intracellular space}}{\text{specific radioactivity in external medium}}$$

(specific radioactivity in the intracellular space was calculated on the assumptions of a tissue density of 1.0 and protein content of 10% wet weight of tissue). Eleven chicks were included in each group.

* $P < 0.02$, ** $P < 0.001$ (Student's t-test, two-tailed).

M: methylanthranilate, W: water.

Table 4.3

Subcellular distribution of fucose incorporation into slices of the right forebrain base of (M) trained and (W) control chicks, 30 mins after training.

Fraction	M	W	M/W x100	t
	nmol/ gm Prot/ hr			
Ext. Med.	3.67 ± 0.34	3.067 ± 0.23	119	1.46
Homog.	56.99 ± 2.47	49.60 ± 1.28	115	2.657**
P1	95.84 ± 2.45	114.0 ± 13.9	84	-1.286
P2	39.46 ± 0.84	39.44 ± 1.13	100	0.014
P3	86.96 ± 35.53	75.65 ± 1.69	115	2.022*
S3	253.26 ± 35.53	283.80 ± 22.7	89.40	-0.721

Chicks were trained, tissue prepared and incubated, and subcellular fractionation conducted as described in Materials and Methods. Values represent the mean ± SEM of fucose incorporation in each fraction (n = 6). *P 0.05, **P 0.01 (Student's t-test, one-tailed).

Ultrastructural Analysis Of The P3 Fraction

An investigation of the ultrastructural nature of the isolated P3 fraction to determine its exact contents indicated that it consisted of membrane fragments. Figure 4.7 shows two electron micrographs of the fixed fraction. Figure 4.7A is a micrograph of magnification of 45K while Figure 4.7B shows greater detail at 70K. It is apparent that the P3 fraction is comprised mainly of membrane fragments and vesicles (M) with some evidence of ribosomes (R), fragments of rough endoplasmic reticulum and golgi. There is also some evidence of synaptosome contamination (S), with the presence of synaptic vesicles (V) and post-synaptic densities (P).

FIG 4.7A

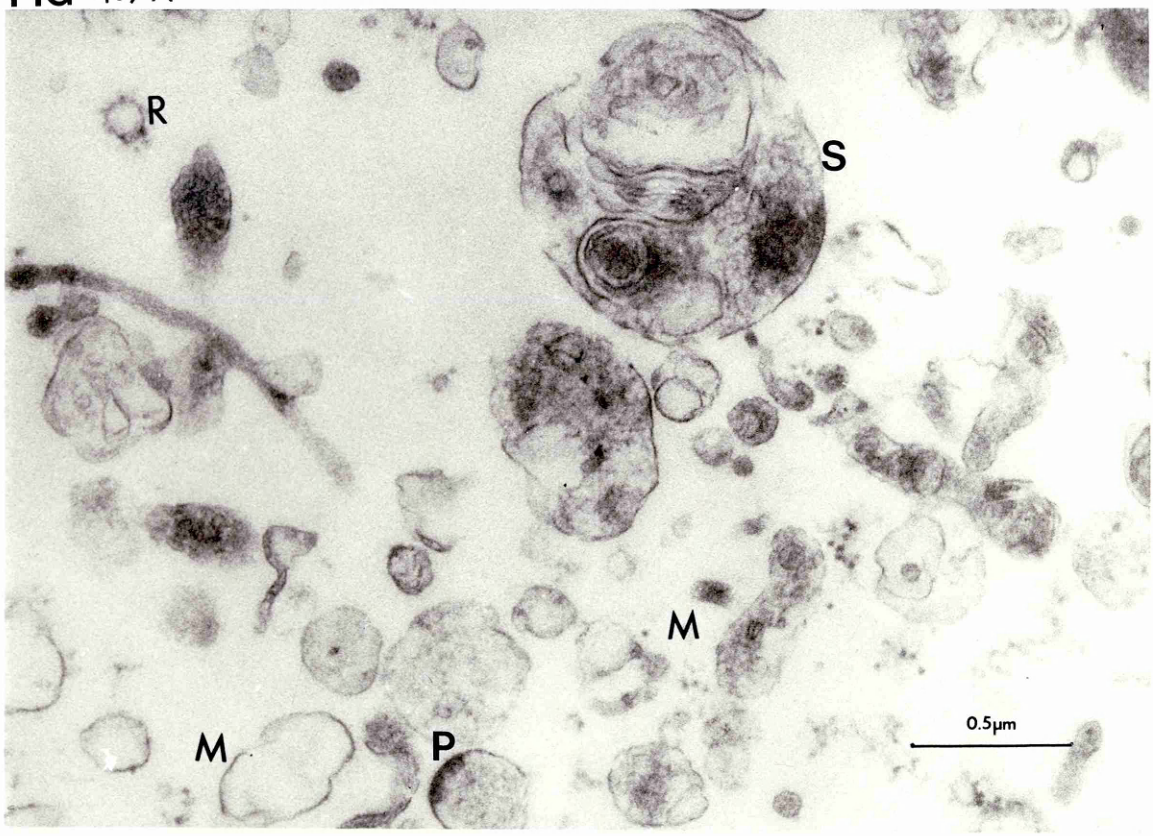
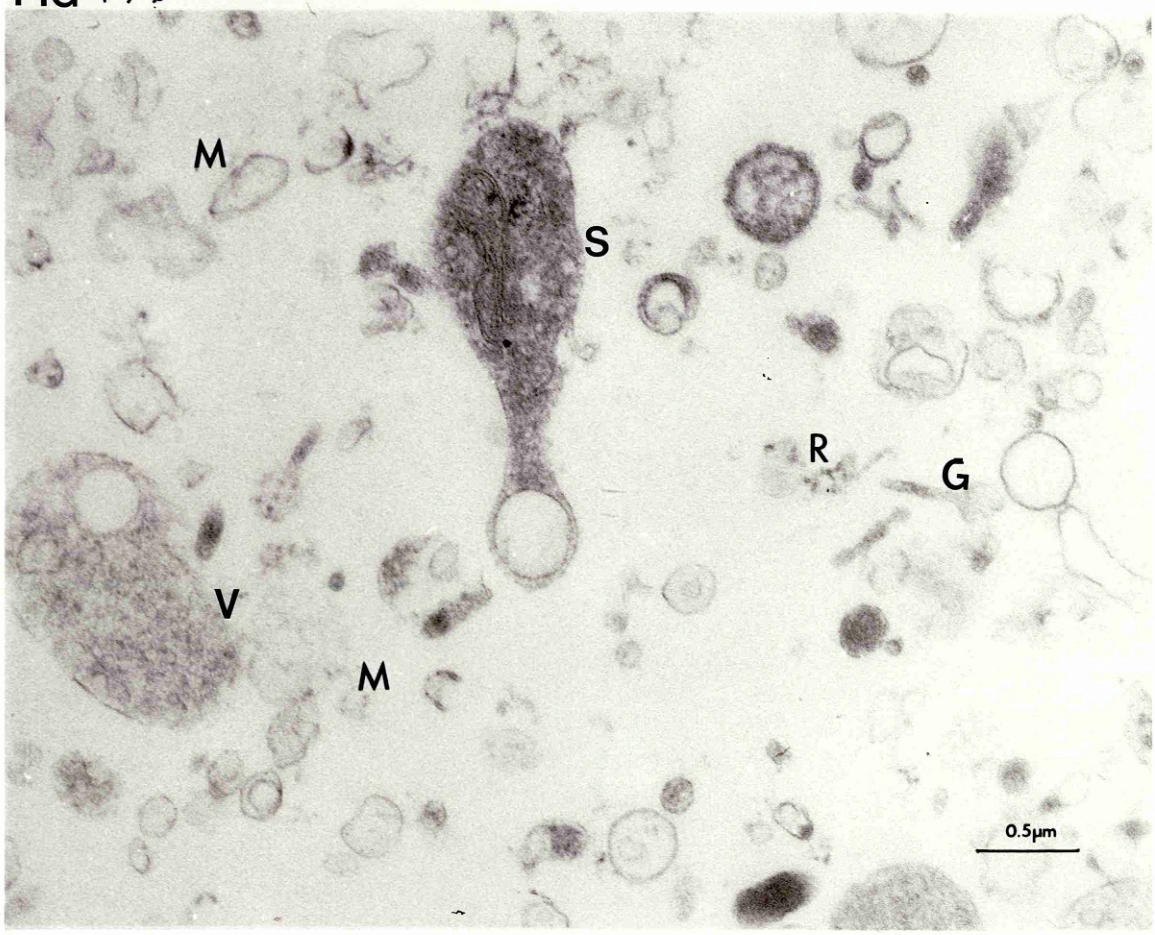


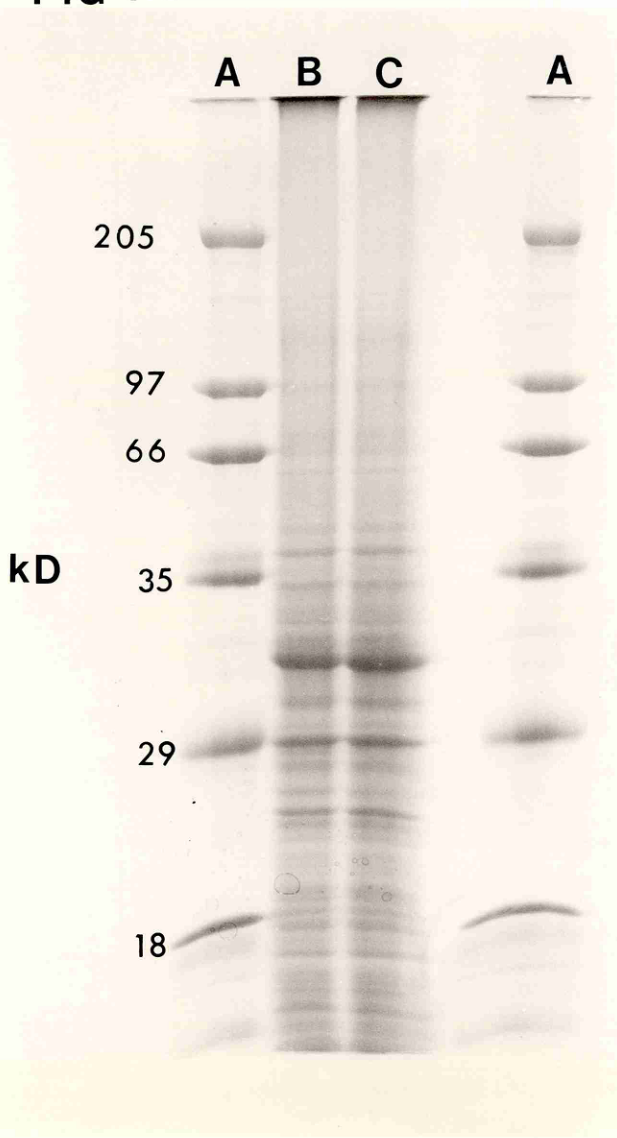
FIG 4.7β



Electrophoretic Analysis Of The P3 Fraction

Figure 4.8 is a photograph of a gel showing 3 protein tracks: (A) molecular weight markers, (B) the P3 fraction (100 ug prot) from an (M) trained chick and (C) the P3 fraction (100 ug prot) from a (W) control chick. It is evident from the photograph that the P3 fraction contains a wide range of molecular weight proteins. On analysis of quantitative densitometrical scans, no difference in absorbance was noted in any of the protein stained bands in the two tracks. Figure 4.9A shows a densitometric scan of the electrophoresed P3 fraction from a (W) control chick, while Figure 4.9B is a scan from a sample from a (M) trained chick.

FIG 4.8



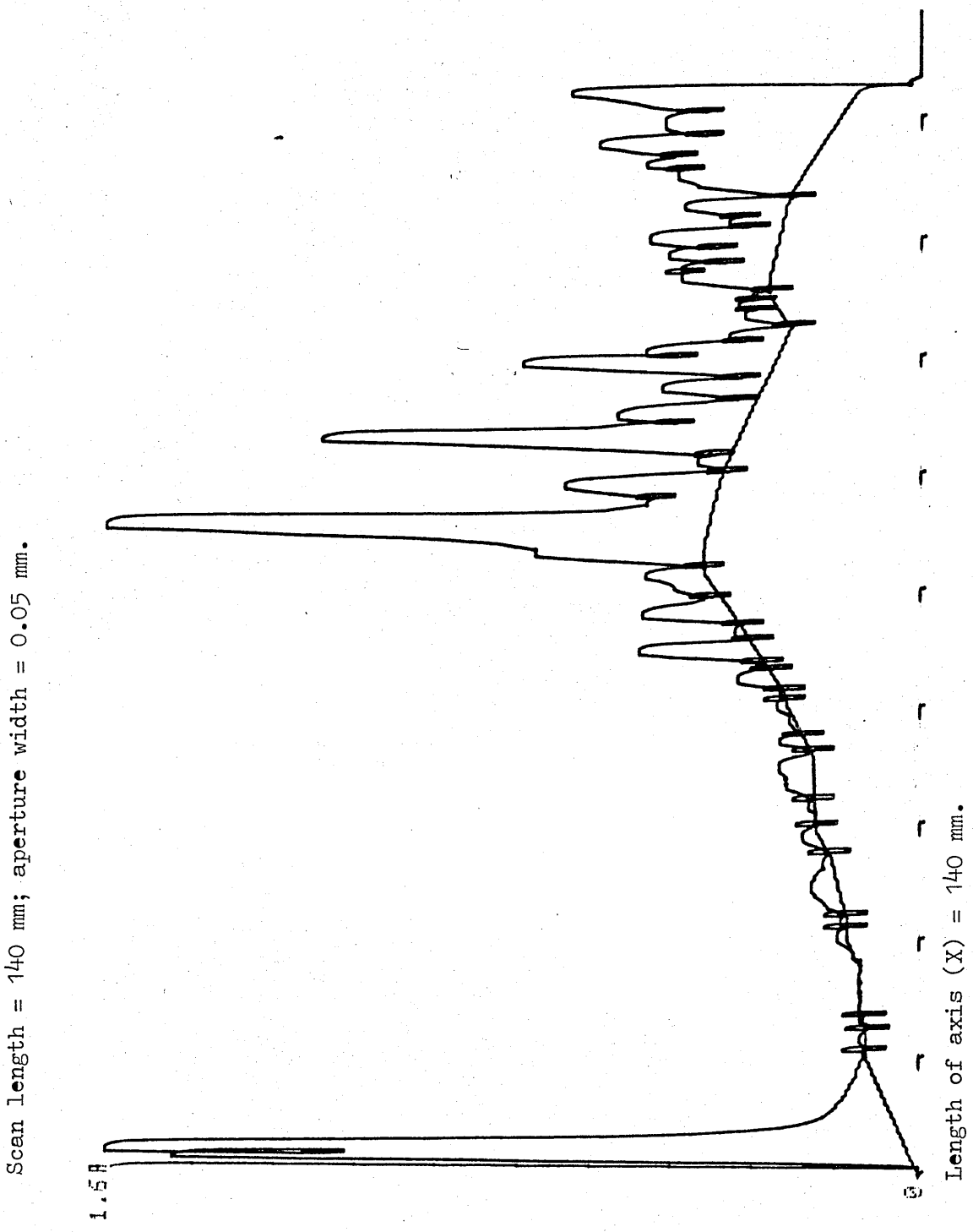


Figure 4.9A. Densitometric scan of electrophoresed P3 fraction of (W) control chick forebrain slices (100 ug protein).

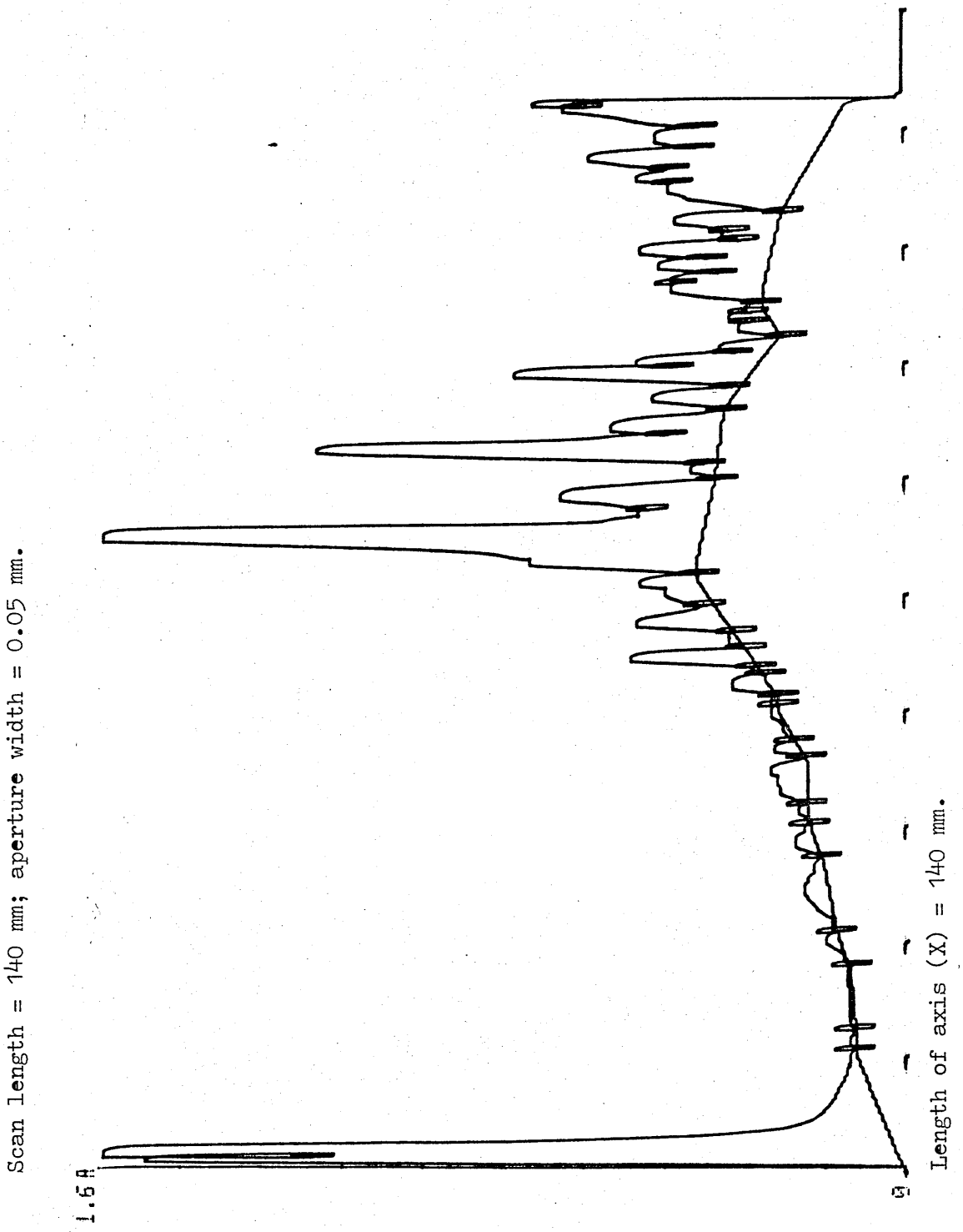


Figure 4.9B. Densitometric scan of electrophoresed P3 fraction of (M) trained chick forebrain slices (100 ug protein).

Each gel track was then sliced into 2mm slices and radioactivity determined. The total number of counts recovered from each gel track as a percentage of total counts put on was approximately 50%, and this was found in each gel analysis. Figure 4.10 shows the number of counts above background obtained from each 2mm slice gel when 400 ug protein of (M) trained (Figure 4.10B) and (W) control tissue (Figure 4.10A) was electrophoresed (experiment 1). The greatest number of counts recorded for any single slice was 168 dpm above background. The total number of counts recovered from the (M) trained tissue track was greater than that obtained from the (W) control track. To obtain an accurate analysis of the number of counts in each gel track, the number of dpm recovered in each slice was expressed as a percentage of total dpm recovered from the track. Figure 4.11 shows this data; Figure 4.11A shows the data for the (W) control chick and Figure 4.11B, data for the (M) trained chick. There was an increase in the amount of labelled fucose in slices (fraction numbers) 15 - 24 in the tissue from the (M) trained chick compared to the (W) control. The protein which was maximally labelled (by 40%) was 100K, and there was an overall increase in labelling of proteins with molecular weights 120K - 82K, as determined by molecular weight markers. Figure 4.12 shows the results of a second experiment in which 100 ug protein of each sample; (M) trained (Figure 4.12B) and (W) control (Figure 4.12A), were electrophoresed. In this experiment, the greatest number of counts obtained above background for any single slice was 60 dpm. The total number of counts recovered from the (W) control tissue was greater than that obtained from the (M) trained tissue. However, on analysis of the number of counts in each gel slice as a percentage of total counts in the gel track (Figure 4.13A (W) control, Figure 4.12B (M) trained), there was an increase in the fucose incorporation in the (M) trained compared to the (W) control in two specific regions.

These corresponded to proteins of molecular weight in the range of 120K - 82K, with the greatest labelling occurring on 100K (as in experiment 1), and proteins with molecular weight 62K - 31K. Duplicate samples of each P3 fraction from this experiment were electrophoresed on the same gel, and similar results were obtained from both samples. However, in a third experiment, electrophoresis of the P3 fraction from another (M) trained and (W) control chick indicated there was no difference in fucose incorporation in any molecular weight band. Thus, increased labelling of proteins in the molecular weight range 120K - 82K was found in 2 out of 3 separate experiments, while a further increase in the proteins of molecular weight 62K - 31K was found in one experiment (each experiment consisting of the P3 fraction of 1 (M) trained and 1 (W) control chick).

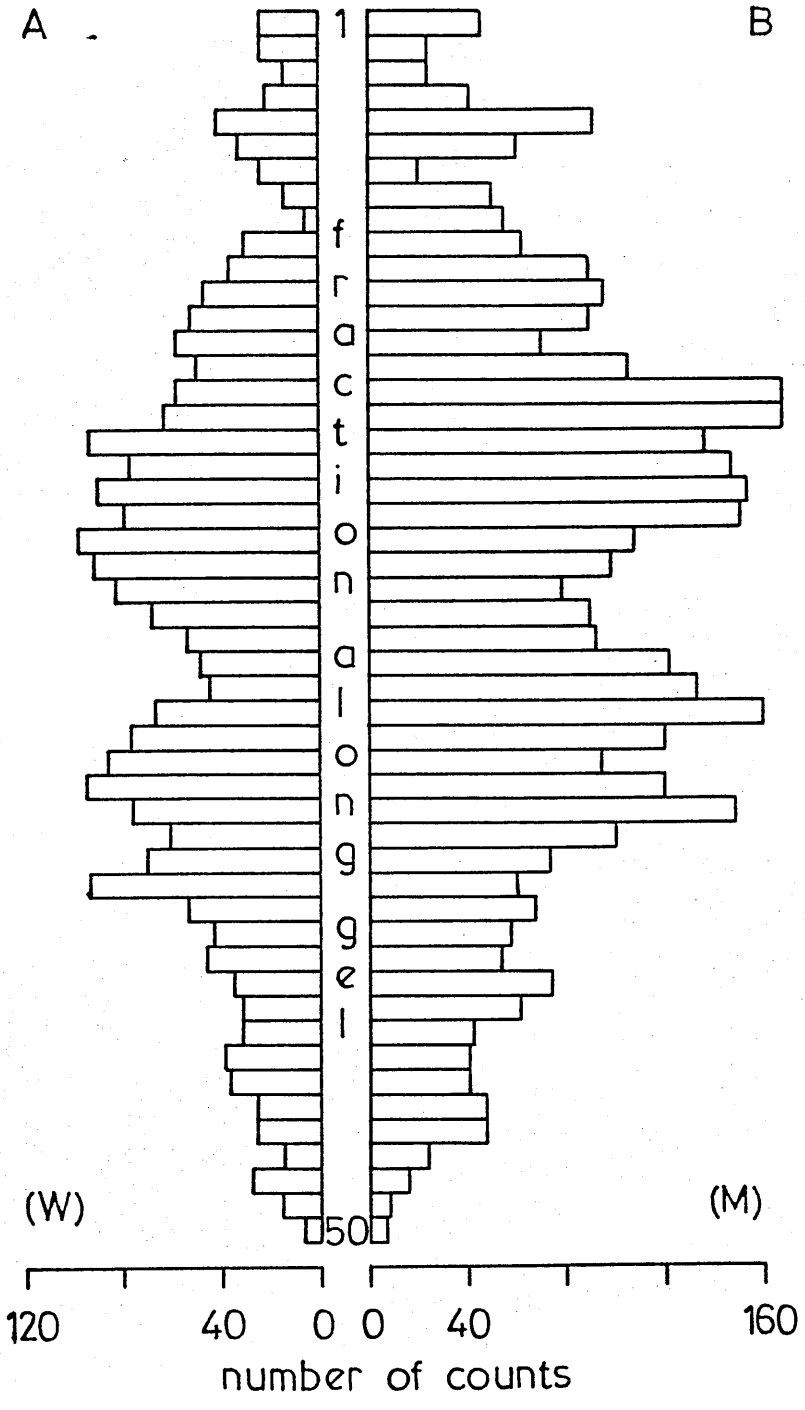


Figure 4.10. The number of dpm's recovered from each gel fraction (2 mm gel slice) from the P3 fraction (400 ug protein) of (W) control chick (A) and (M) trained chick (B). Fifty gel fractions were obtained from each track. This represents Experiment 1.

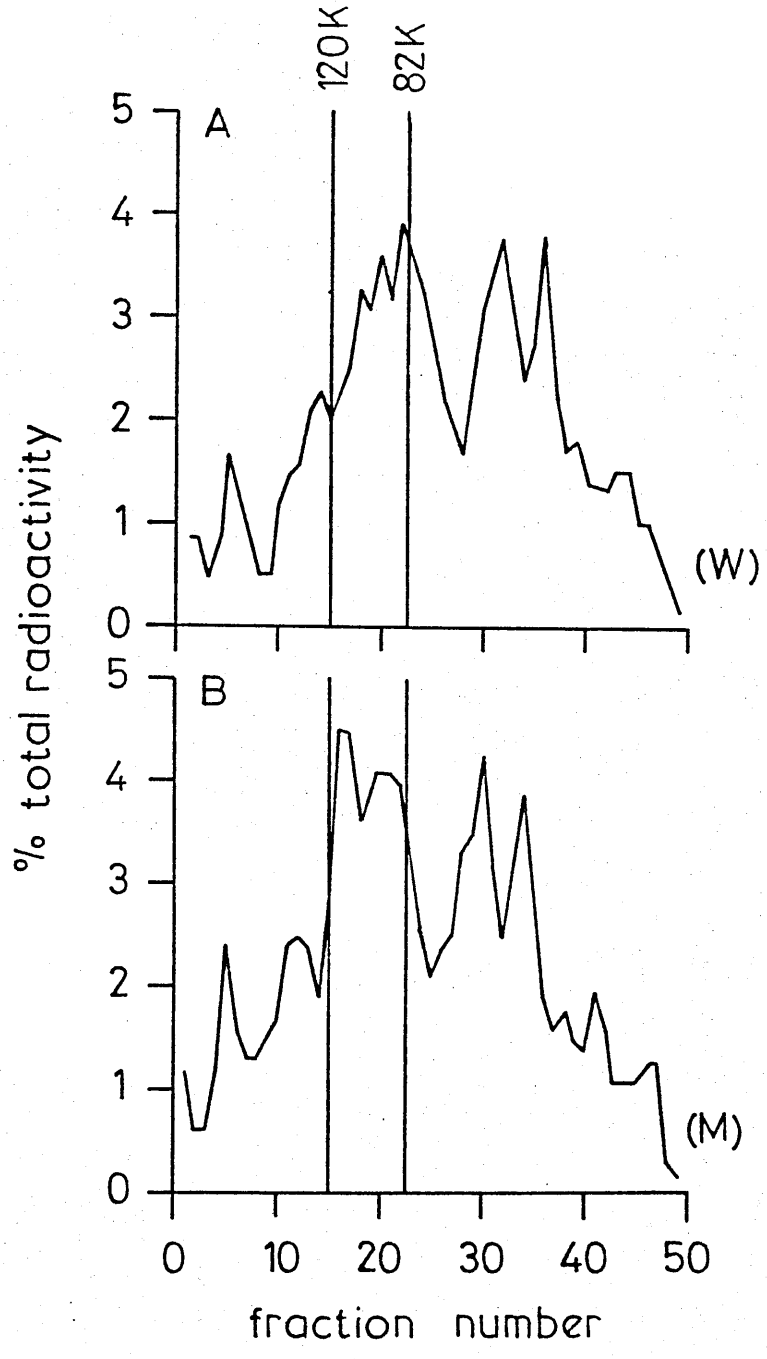


Figure 4.11. This Figure shows the data presented in Figure 4.10. (as counts in each gel fraction) as a percentage of total counts recovered from the whole gel track (400 ug protein). A: % counts from (W) control chick; B: % counts from (M) trained chick. There was an increase in the amount of radioactivity in the molecular band region 120-82K in the (M) trained over the (W) control sample.

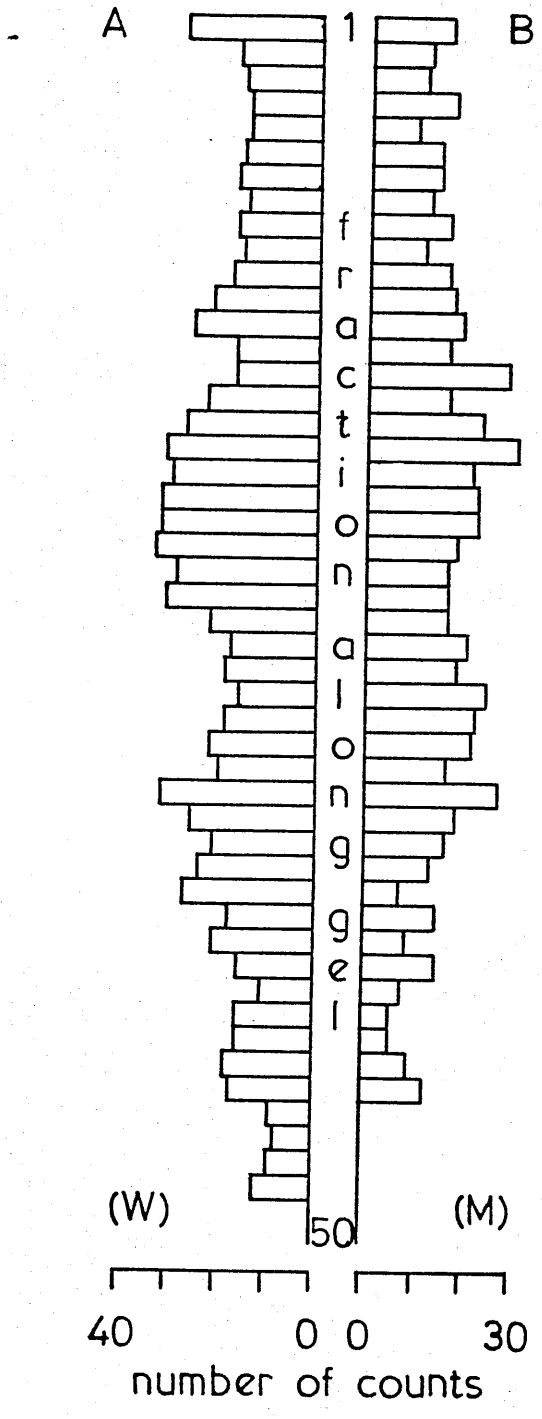


Figure 4.12. The number of dpm's recovered from each gel fraction (2 mm gel slice) from the P3 fraction (100 ug protein) of (W) control chick (A) and (M) trained chick (B). Forty eight fractions were obtained from the (W) P3 fraction, and 44 from the (M) fraction. This represents Experiment 2.

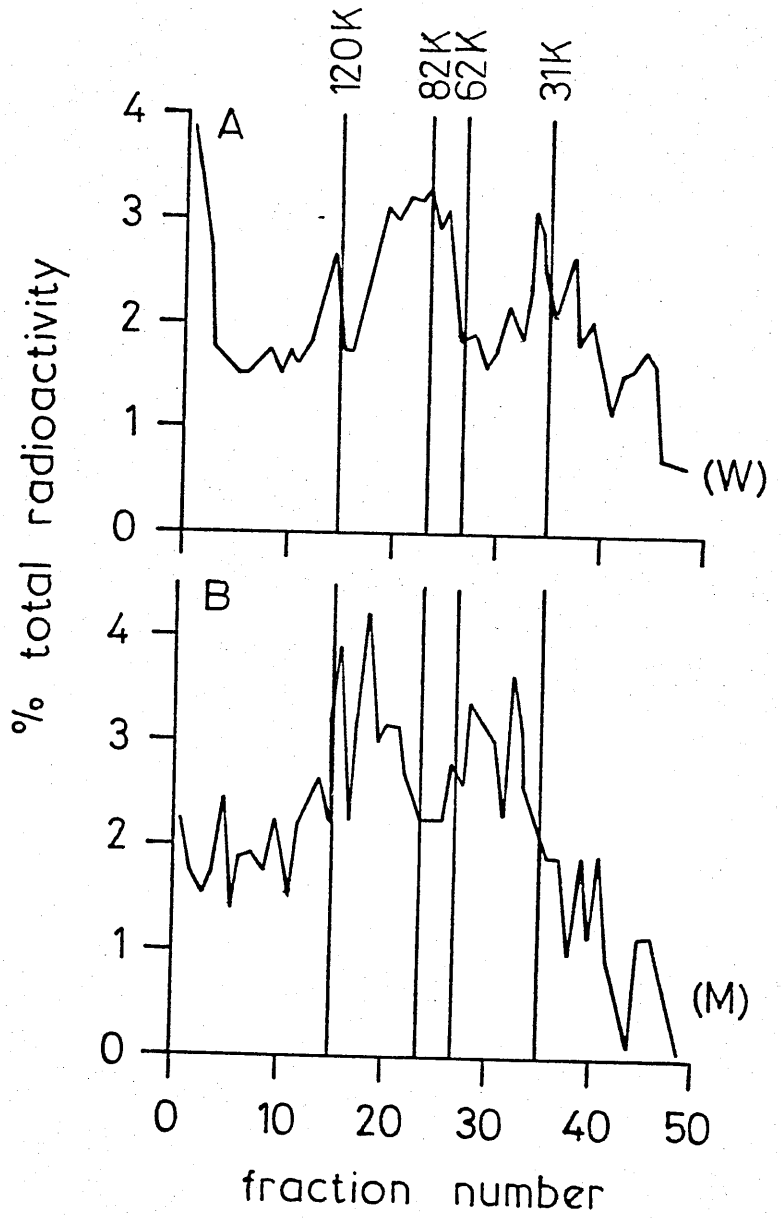


Figure 4.13. This Figure shows the data presented in Figure 4.12. (as counts in each gel fraction) as a percentage of total counts recovered from the whole gel track (100 ug protein). A: % counts from (W) control chick; B: % counts from (M) trained chick. There was an increase in the amount of radioactivity in two molecular band regions in the (M) trained over the (W) control sample. This indicates increased fucose labelling of proteins in the molecular band widths 120-82K and 62-31K.

Effect of Neurotransmitters and EGTA on Fucose Incorporation

In order to determine whether the mechanism of increased fucose incorporation might be associated with a neurotransmitter effect, fucose incorporation was determined when a range of neurotransmitters were added to the incubation medium. Table 4.4 shows that when noradrenaline was added to the incubation medium, the highest concentration used (10 mM) caused a 23% inhibition of fucose incorporation compared with control values. This was a significant decrease ($P<0.05$) and a similar significant decrease was also evident with a concentration 100 fold lower (0.1 mM). With the lowest concentration used (0.001 mM), incorporation values were similar to those of control. The same general effect was noted with dopamine, a 10 mM concentration resulting in a 35% ($P<0.01$) inhibition of control fucose incorporation levels. Reducing the concentration to 0.05 mM brought activity back to control values. Various concentrations of 5-hydroxy-tryptamine added to the incubation medium had no effect on fucose incorporation. With acetylcholine, decreasing the concentration to (0.05 mM) led to an inhibition of 26% ($P>0.05$) in fucose incorporation values.

Table 4.4

The Effect of Some Neurotransmitters on Fucose Incorporation *in vitro*.

	Transmitters			
	NAD	DA	5-HT	ACH
Conc (mM)	nmol/gm prot/hr			
Control	40.56 -1.64	42.33 - 1.30	42.23 - 1.30	52.15 - 4.86
10.0	30.33 - 2.4*	27.67 - 2.67*	----------	41.33 - 2.14
0.5	36.67 - 1.94	35.65 - 2.36	----------	40.23 - 3.29
0.1	30.34 - 2.1*	34.51 - 2.17	42.33 - 1.64	39.22 - 1.77
0.05	37.34 - 2.46	39.17 -7.52	43.33 - 1.94	38.66 - 2.21*
0.001	40.84 - 3.17	----------	39.44 - 2.98	----------

Forebrain slices were prepared and incubated as described in Materials and Methods. 100 ul of neurotransmitter, in a range of concentrations, was added to the medium and fucose incorporation into an acid-insoluble fraction determined. Values represent mean $\pm$ SEM (n = 3).
*$P < 0.01$ (Student's t-test, two-tailed).

Effect on fucose incorporation when slices were incubated in a calcium-free medium

When slices were incubated in a calcium-free medium, there was no difference in fucose incorporation rates when compared to incorporation rates determined under control conditions. Table 4.5 shows the effect of a calcium-free medium on fucose incorporation rates in chick fore brain slices, and fucose incorporation in slices incubated in normal medium containing 2.6 mM calcium.

Table 4.5

The Effect of a calcium-free medium on Fucose Incorporation in vitro.

	nmol/g prot/hr
Control Medium	36.32 +/- 1.08
Calcium-Free	37.84 +/- 2.41

Values represent mean ± SEM of three determinations of fucose incorporation in slices in control medium (2.6 mM calcium) and calcium-free medium. Incubations were run in parallel.

4.3 DISCUSSION

The advantages of *in vitro* techniques have been apparent for many years, especially in the study of protein metabolism in mammalian tissue (reviewed by Heald *et al* 1960). The majority of these experiments have employed labelled precursors, in particular amino acids, to detect changes in metabolism. They have the important advantage over *in vivo* work in that one can apply the label directly to the area of interest and thus the amount of radioactivity required for each experiment is significantly reduced; there is therefore an increase in the efficiency of the system. Also, the attendant problems of differential rates of absorption and cellular uptake are diminished. This is particularly obvious in comparing this *in vitro* fucose incorporation system to the *in vivo* system investigated by Sukumar & Rose (1980). The absolute amount of radioactivity detectable in this study, as a percentage of radioactive fucose added, is much higher than that found *in vivo* by Sukumar *et al* (1980). *In vivo*, after an injection of 7.4 MBq/ml of ^{3}H-fucose, specific activity 6.7 TBq/mmol, values of fucose incorporation into the particulate fraction were 15,000 dpm/mg prot. This corresponds to a rate of 1.0 umol/g prot for a 3 hr period. In contrast, in this *in vitro* study, incubating forebrain slices in a medium containing 3.7 KBq/ml L-^{14}C-fucose, specific radioactivity 2.2 GBq/mmol, for a 3 hr period gave a rate of incorporation of 111 nmol/g prot.

Doubts as to the reliability of methods using radioactive precursors of macromolecules as tracers in *in vivo* experiments in drawing conclusions about the chemical correlates of behaviour were emphasised in a commentary by Glasman (1974). He claimed that changes in the amounts of radioactive precursor in individual cells or areas of the nervous system can occur because of localized changes in blood

flow, changes in cellular permeability to the radioactive compound, changes in endogenous rate of synthesis and localized differences due to unavoidable variations in the injection. These problems have also been considered by Popov et al (1976a, 1980) in in vivo studies, in which they investigated training-related increases in fucose incorporation in rats. However, using an in vitro system, one does not encounter these problems.

But before one can say that in vitro work is indicative of the in vivo situation, one must ensure that the integrity of the tissue and composition of the physiological medium are comparable to those found in vivo. In this study, I used a mechanical device to cut the forebrain slices in preference to a manual method, because of greater speed and accuracy in obtaining slices of uniform thickness. The electron micrographs do show, however, that there is some tissue damage in a 50 um zone internal to the cut surface. Other studies in which histological analysis of brain slices cut mechanically were investigated have also revealed that there is a substantial amount of dead or damaged tissue along the cut surface. Tissues investigated include hippocampal (Frotscher et al 1981), cerebellar (Garthwaite et al 1979) and neostriatal slices (Bak et al 1980). Garthwaite et al (1979) also carried out a comparative study on the ultrastructure of hand-cut and mechanically chopped (McIlwain tissue chopper) slices from adult rat cerebellum. After 2 hr of incubation, they found that the state of the hand sliced adult cerebella was much superior to that of the chopped material. However they found that there was little difference in the state of preservation of immature cerebella, when hand-cut and mechanically chopped tissue were compared.

To ensure that the incubation parameters used in this study did not lead to appreciable distortion of internal cellular structure of the slices, electron micrographs of pre- and post-incubated tissue were examined. Bak et al (1980) defined a number of criteria which can be used in assesing the degree of intact morphology of tissue in vitro. These include (a) lack of swelling or shrinkage of cells (average diameter 15 - 29 um), (b) homogenous nucleoplasm with distinct nucleoli, (c) mitochondria with parallel cristae and dense mitochondrial matrix, (d) manifest cell organelles (endoplasmic reticulum, Golgi apparatus) in the cytoplasmic matrix and (e) smooth and uninterrupted cell and nuclear membranes. These characters can be seen in the pre-incubated tissue as is evident from Figure 4.7. However, there was some evidence of mitochodrial swelling in slices after incubation, which is probably attributable to osmotic imbalances and general insult to the tissue inflicted by the mechanical chopper (Figure 4.7B). In a study on protein synthesis in vitro in chick forebrain slices, Schliebs et al (1985) prepared slices by a similar method. They carried out an extensive study, comparing the ultrastructure of slices in vitro to that of slices prepared from perfused brain tissue. They concluded that the poor preservation of slice tissue in vitro was probably due to the physical damage from the chopping process rather than any physiological deterioration due to incubation, as the appearance of slices before and after 90 minutes of incubation was similar.

Slices were cut to a thickness of 400 um to ensure adequate oxygenation and diffusion of metabolites. In a study investigating incubation of rat hippocampal slices of various thickness, Whittingham et al (1984) showed that with increasing slice thickness, up to 700 um, there was evidence of increasingly large areas of anoxic tissue which contributed to an overall reduction in metabolite levels. In my

study, adequate oxygenation of the medium was ensured by perfusing with 95% O_2, 5% CO_2 for 20 min before pre-incubation and incubation proper. To ensure adequate diffusion of substrates, the slices were shaken in a water-bath throughout the incubation period. It is well known that between all types of lipid/water interfaces there exists an aqueous layer which is very slowly renewed and which can inhibit adequate oxygen diffusion. Shaking reduces this so called 'unstirred layer' (Korpi & Oja 1984). The medium used, a Hepes buffer pH 7.4 was identical to that used by Schliebs et al (1985), in which an active protein synthesising system in chick forebrain slices was investigated. In addition, Hepes has been found to be superior to Tris as a buffering system for in vitro protein synthesis systems, as investigated by Hustor et al (1970) in a study on rat liver.

This is the first time, to my knowledge, that an active fucose incorporation system has been investigated in chick forebrain slices.

Fucose incorporation was maximal at 42 C, which agrees well with studies by Liu et al (1975), which showed that this was the optimum temperature for in vitro protein synthesis in chick brain tissue after a 3 hr incubation period. This is not surprising, as the body temperature of the chick is 42 C (Weiss et al 1982). Incorporation into the TCA insoluble fraction was linear over the 3 hr of incubation (Figure 4.2), but the absolute values of fucose incorporation varied by as much as 20% between different chick hatches. Equilibration with the internal medium was achieved after 90 min of incubation (Figure 4.3). (Calculation of fucose incorporation in the intracellular space were based on the assumptions of a tissue density of 1, and that the protein content was 10% of wet weight). All sugars are transported into the cell via a system of facilitated transport, such that the ratio of internal:external sugar concentration is 1. However in this

in vitro system, the maximum internal fucose concentration recorded never exceeded 70% that of the external medium. This was probably due to minor tissue damage and distortion during incubation.

Data from the studies of fucose incorporation rates in vitro in slices prepared from methylanthranilate (M) trained and water (W) control birds show that, 30 min after training there was a 16% increase in fucose incorporation in the right base of trained birds; which was not evident 24 hr after training (Table 4.1A 4.1B respectively) (McCabe & Rose in press). This increase cannot be attributed to an increased uptake of fucose into the intracellular space, as a significant uptake was observed in the R.B., L.B. and R.R. in trained birds over controls, while a significant incorporation of fucose into the acid insoluble fraction was only evident in the right base of trained chicks. This is further confirmed by the in vivo fucose incorporation study, in which significant increases in fucose incorporation into the particulate fraction of (M) trained chicks over (W) controls were observed. However, no such change was found in the soluble fraction, which rules out the possibility that the observed elevation was an artifactual consequence of changed precursor pool availability (Sukumar et al 1980). The increased uptake may be due to either an increased intracellular pool as a result of minor differential tissue swelling (previously noted in the ultrastructural analysis), a factor which was also evident and discussed with respect to protein synthesis in vitro (Schliebs et al 1985). An alternative explanation might be associated with stress-induced uptake of substrates, including fucose, in response to tissue damage. That there was an increase in fucose uptake in three forebrain regions of (M) trained compared to the (W) control chicks, indicated that this may be a specific training-related increase. Perhaps the increased uptake observed as a result of

training is associated with enhanced facilitated transport, mediated by an increase in the concentration of membrane bound carriers.

Subcellular fractionation studies of the right forebrain base of trained and control chicks showed that the increase in fucose incorporation was localized to the P3 fraction (Table 4.3). This microsomal fraction, examined using electron microscopy, contained in addition to endoplasmic reticulum, such minor components as Golgi apparatus, plasma membranes and fragments of mitochondria, lysosomes and there was also some evidence of synaptosomal contamination (Figure 5.7). That the increase in fucosylation as a result of training was not found in the P2 fraction (an enriched synaptosomal fraction) would indicate that this contamination can be ignored. The sites of protein glycosylation within the cell have been well documented (Hughes 1983) and have been discussed earlier (Chapter 2). The endoplasmic reticulum and Golgi apparatus are proposed to be the sites involved in the packaging of the glycan moiety to the protein, and in particular, they are the sites of addition of the terminal sugar (of which fucose is one) to the completed glycoprotein. This is the mechanism of production of both secretory and membrane-bound glycoproteins, in particular those of the plasma membrane. Location of the fucosylated proteins in this study to a microsomal fraction suggest that these glycoproteins have not reached their final destination, the plasma membrane, where 80% of brain glycoproteins are found (Brunngraber 1972). In an _in vivo_ fucose incorporation study, Burgoyne & Rose (1980a) found that after an intracranial injection of labelled fucose, an increase in incorporation in trained compared to control birds was found in a synaptic membrane fraction 3 hours after training. Perhaps if incubation was carried out for a longer period, I would have found the increased fucose incorporation in a similar synaptosomal fraction _in vitro_. Unfortunately this was not possible, as with incubation

times longer than 3 hr there was appreciable loss of the medium by evaporation (the stoppered flasks were not airtight). On reconsideration of this experimental method, perhaps incubation could be prolonged (using sealed flasks), but one has to remember that, in working with isolated tissue preparations a compromise between incubation period and maintenance of tissue integrity must be reached. The location of the observed fucose incorporation is in agreement with results obtained from a time course study of *in vivo* fucose incorporation in day-old chicks. Murakami *et al* (1985) found that 3 hr after an intracranial injection of U-L-^{14}C fucose most of the label was found in a similarly prepared P3 fraction.

On further analysis of the P3 fraction of the forebrain base of (M) trained and (W) control chicks by S.D.S. gel electrophoresis, an increase in fucose incorporation was noted in proteins with molecular weights ranging from 120K - 82K and, in particular, in a 100K protein (Figure 4.11). However, the molecular weights of glycoproteins are not truly represented by polyacrylamide gel electrophoresis because they do not bind S.D.S. to the same degree as proteins. Consequently, it may be that their molecular weight estimates are too high (Mahadik *et al* 1976). Burgoyne & Rose (1980) reported that on analysis of the synaptic membrane fraction from trained and control chicks, the increased fucose incorporation observed *in vivo* was not limited to any particular glycopeptide, but that an increase occurred in all of the nine electrophoretically separated proteins, with molecular weights ranging from 162K - 23K. The increases in fucose incorporation as a result of training found in this study, in the molecular band region (120K - 82K), was replicated in 2 out of 3 experiments, while a further increase in incorporation in proteins of molecular band width 62K - 31K was evident in one experiment. There was no difference however, in the absorbance of any protein band when

all the gels were densitometrically scanned. This may indicate that the increase in fucose label in specific proteins as a result of training is due to an increased fucose incorporation of proteins already present, rather than an increase in fucosylation of proteins synthesized de novo. However, one must consider whether the scanner being used is sufficiently sensitive to pick up minor protein changes if they do exist. Although replication of the increased labelling of certain protein bands as a result of training was not 100%, I think one can explain this inability from both a behavioural and a biochemical perspective. As discussed previously, there is a great deal of variability between chicks during training and as each gel experiment involved the analysis of only one trained and one control chick, the problems of behavioural variability could not be reduced by working with large sample sizes (referred to in Chapter 2). From a biochemical stand-point, the major problem was an inability to get high concentrations of radioactive label in each sample. For my gel analysis, the slices were incubated with labelled fucose which was 100 times the specific radioactivity used in other experimental procedures, but only 50% of total counts put on the gel was recovered from each gel track. The highest number of counts recorded for any single gel slice was only 60 counts above background when 100 ug protein samples were electrophoresed. This problem was resolved when 400 ug samples were available for gel analysis, in which up to 169 counts above background were detected in some slices. The major disadvantage with this method is the limitation of the protein content of the TCA precipitated P3 fraction, and thus the inability to replicate electrophoresis of samples. Many other investigators have also encountered this problem of not achieving high enough radioactivity in samples for gel analysis. Quarles & Brady (1971), in similar electrophoretic studies of protein, found that after applying

0.5 mg protein samples to each gel, the sensitivity of the method was still limited by the amount of radioactivity incorporated.

From these results it might be suggested that training causes an increased production and hence fucosylation of particular proteins with molecular weights in the range 120K - 82K, and in particular 100K (and perhaps 62K - 31K), or alternatively, that training causes an independent increase in fucosylation of proteins in this molecular weight range. This is the first study to report a training related increase in proteins of this molecular weight. Previous studies investigating the involvement of a brain protein (found initially in the brain and originally thought to be brain specific), S-100, in learning and memory are numerous (reviewed by Hyden 1976). S-100 is an acidic protein with a molecular weight of 21,000 (Moore 1973; Calissano 1973), and constitutes 0.1 - 0.2% of the total brain soluble protein (Moore 1973). On training rats to reverse handedness, the amount of soluble S-100 increases in hippocampal nerve cells by 20% and the incorporation of labelled precursors increases by 300% (Hyden & Lange 1970). Injection of a specific antiserum against the S-100 protein produced a concomitant defecit in learning, further substantiating its involvement in the learning process. On further investigation of the location of the antiserum, a high concentration of S-100 was found in the post-synaptic plate (Hyden & Lange 1970). Shashoua et al (1984) have recently reported that, on sampling cerebrospinal fluid (C.S.F.) from anaesthetized rats, there is evidence of the release of S-100 protein, which may indicate that it is synthesised in some other cell (perhaps glial cells) and consequently transfered to neurones, the specific site of action. Hyden (1976) has formulated a hypothesis on the involvement of S-100 protein in the cell biology of learning and memory. It includes the idea that the neuronal membrane and, specifically, the post-synaptic

areas are differentiated by the S-100 protein. It's conformational state is dependent on calcium concentration, a characteristic shared by the actin-like filaments of the membrane associated network. Thus changes in intracellular calcium may lead to morphological changes in neuronal membranes , via S-100, leading to molecular and structural differentiation of neurones. However, there is growing skepticism about the involvement of S-100 protein in neuronal plasticity. It was originally believed to be unique to the nervous system, but it has recently been found in other structures, including the skin, lymphoid organs, and in the lens and cornea of the rabbit eye (reviewed by Michetti et al 1985).

Later experiments by Shashoua (1979), investigating the biochemical changes associated with training goldfish on a vestibulomotor adaption task, indicated that changes in the metabolism of two proteins called ependymin B and Y are involved. These proteins are two of the most abundant found in the extracellular fluid of the goldfish brain. Antisera to B and Y, injected into the brain shortly after training produced amnesia for the training task (Shashoua & Moore 1980), and later experiments (Shashoua 1981) indicate that these proteins become rapidly labelled and secreted into the cerebrospinal fluid. These experiments suggest that protein activated by a training experience might be exerting effects at loci remote from the cells of origin. To investigate if there was any evidence of glycoprotein secretion from the chick forebrain slices in this study, as a result of training, radioactivity in the T.C.A. precipitable portion of an aliquot of the external medium was analysed. However, only 6% of the total T.C.A. precipitable radioactivity recovered from the slices was found, and there was no difference in the medium radioactivity from slices of trained and control chicks. These findings compare well with those of Benowitz & Shashoua (1979), who examined the

incorporation of labelled valine in proteins of the extracellular fluid (ECF) and cytoplasm of chicks trained on a one-trial passive avoidance task (similar to the training paradigm used in this thesis). They found that there was no difference in the incorporation of label into any protein in the ECF or cytoplasmic fractions which could be correlated with the training procedure.

This in vitro investigation replicates the finding of an increased fucose incorporation in vivo of Rose & Harding (1984), in which a 26% increase in fucose incorporation was localized to the forebrain base of trained chicks compared to controls, 3.75 hr after training. In these in-vivo studies, lateralization was not examined. The increase in this study was found in the right forebrain base and may be related to a 14% increase in fucokinase activity in the same region of trained chicks one hour after training (Lossner & Rose 1984). However, this increase is contrary to the training -related fucokinase activity reported in this thesis, with increased fucokinase activity in the left forebrain base at the same time after training. (I have referred to this disparity in results in the previous Chapter). The forebrain base has been sited as a locus in which other biochemical changes have been noted as a result of training chicks on P.A.L. (as discussed in Chapter 2). In particular, an increased uptake of 2-DG has been found the lobus parolofactorius, a nucleus located in the base region (Kossut & Rose 1984; Rose & Csillag 1985).

In vivo, the increased fucose incorporation persisted for up to 24 hr (Sukumar et al 1984), but no increase was found in vitro when slices from chicks tested after a 24 hr period were incubated. However, one cannot compare these data, as the in vivo experiments involved an injection of label one hour prior to training. Data on fucokinase activity also indicate that there was no increase 24 hr

after training (Chapter 3).

A study to determine if increased fucose incorporation could be elicited by a range of monoamine or a cholinergic transmitter/s was investigated. My results indicate that noradrenaline and dopamine cause a significant inhibition of fucose incorporation, at a concentration of 10 mM. The effect of a range of concentrations was studied but dose response curves were not apparent. Various concentrations of 5-hydroxy-tryptamine had no effect on fucose incorporation rates, while decreasing concentrations of acetylcholine caused a non-significant inhibition of fucose incorporation. These studies were stimulated by the work of Jork et al (1982), in which dopamine at a concentration of 50 uM, and more specifically, apomorphine elicited increased fucose incorporation in rat hippocampal slices, which replicated the increase observed on training rats on a learning task (Popov et al 1976a). Further to these observations, Jork et al (1982) found that administration of intra-hippocampal injections of apomorphine, after the aquisition of a brightness discrimination task, increased the period of retention for the task. They also found that there was an increased fucose incorporation in trained rats receiving an injection of apomorphine over trained animals which had received an intra-hippocampal control injection of solvent. Longstaff & Rose (1981) reported a transient increase in muscarinic receptors after training chicks on P.A.L., so it was interesting to determine if this observed increase was linked to the reported increase in fucose incorporation, and if it was mediated via acetylcholine. However when acetylcholine was added to the incubation medium decreasing concentrations did not lead to significant inhibition of fucose incorporation. These results compare well with those of Matthies (1979), in which neither muscarinergic nor nicotinergic cholinergic agonists altered incorporation of either

leucine or fucose in hippocampal slices, as observed in training experiments. However, there are contrary reports in the literature which do suggest an involvement of the cholinergic system in learning and memory. Sandberg et al (1984) have reported a significant impairment in the aquisition and retention of a step-down avoidance task in rats with striatal lesions induced by the cholinergic neurotoxin AF64A. They concluded that this observed deficit in learning supports the theory that the striatal cholinergic system is involved in complex behavioural systems. These conclusions are in agreement with studies involving lesions to the cholinergic pathway in the basal forebrain of the rat (Miyamoto et al 1985). In particular, it is interesting to note that cholinergic system lesions have gained immense recent importance in relation to experimental models for Alzheimer's disease and senile dementia (Lippa et al 1980; Strong et al 1980), in that the major defecit in Alzheimer's disease is in the synthesis of Ach, probably in the nucleus basalis (Bowen et al 1973).

The involvement of the neurotransmitters I have studied, in fucose incorporation would seem to indicate that the training related increase in fucose incorporation is not mediated by a neurotransmitter mechanism. That high concentrations (10 mM, higher than physiological concentrations) of noradrenaline and dopamine should give significant inhibitions of fucose incorporation in vitro may suggest that they are acting via inhibitory synapses; high concentrations of dopamine can have an inhibitory effect via pre-synaptic receptors. The inhibitory effect observed with noradrenaline may be associated with an increase in C'AMP, which, as reported in Chapter 3, causes an inhibition of fucokinase activity. This may be tenuous evidence that mechanisms which inhibit fucokinase activity have a similar effect on fucose incorporation, thus one could suggest that there is a link between the two mechanisms.

On analysis of fucokinase activity as reported in Chapter 3, results indicated that increasing concentrations of calcium led to an inhibition of activity. I was therefore interested to determine if fucose incorporation rates *in vitro* were effected by incubation in calcium-free medium, and thus provide a mechanism by which fucokinase activation might be involved in fucose incorporation. However, rates of fucose incorporation in slices incubated in a calcium-free medium did not differ from incorporation values found in slices incubated in the normal incubation medium (2.6 mM Ca^{2+}). This would suggest that if fucokinase is involved in the increase in fucose incorporation as a result of training that changes in calcium concentration are not involved. Perhaps concomittant with an observed increase in fucokinase activity, training results in the activation of fucose pyro-phosphorylase and/or fucosyl transferase, other enzymes involved in the metabolism of fucose into glycoproteins (as discussed in Chapter 3). This hypothesis may be related to the work of the Magdeburg group, who have found that increased fucose incorporation into rat hippocampal proteins (Matthies 1978; Lossner *et al* 1981) is related to the increase in fucokinase activity up to 2 hours after training (Popov *et al* 1983). However, the increased incorporation recorded 6 - 8 hours after training seems to be related to enhanced activity of fucosyl-transferase (Popov *et al* 1983), an enzyme whose activity has not been studied in the chick brain.

In summary, this investigation further consolidates the finding of an increased fucose incorporation as a direct result of training chicks on a passive avoidance task, in that the effect initially found *in vivo* has now been successfully replicated *in vitro*. This is the first time that a training related increase in fucose incorporation has been successfully achieved *in vitro*. This increase in fucose incorporation has been localized to the right forebrain base which is

comparable to the data from in vivo fucose incorporation and also fucokinase activation. Further analysis has shown that the increase is confined to glycoproteins with molecular weights in the range 120K - 82K and in particular 100K. The mechanism of increased fucose incorporation does not seem to be wholly concerned with fucokinase activation (as far as the present experiments indicate), and may indicate the involvement of the other enzymes in the metabolic pathway. It also seems that the increased fucose incorporation is not associated with the effects of the adrenergic or cholinergic transmitters investigated in this study.

These experiments beg the question as to what is happening when increased fucose incorporation occurs as a result of training. Does training cause an increase in protein synthesis de-novo with consequent fucosylation, and hence an overall increase in glycoprotein turnover/production, or, alternatively, does training result in an increased fucosylation of pre-existing proteins, indicating that fucosylation is the single most important biochemical event associated with training/learning? The following Chapter investigates this question.

CHAPTER 5

A STUDY ON FUCOSE INCORPORATION *IN VITRO* AND *IN VIVO*.

The aim of the experiments reported in this Chapter was to further investigate the increase in fucose incorporation *in vivo* and *in vitro*, observed after training chicks on a passive avoidance task (P.A.L.). The *in vitro* studies discussed in the last Chapter indicate that there is an increase in fucose incorporation in the right base one hour after training. It is not clear whether this is associated with an increase in protein synthesis *de novo*, or is an independent biochemical effect occurring on pre-synthesized proteins.

Because of the importance of protein synthesis in cellular regulatory processes, it has long seemed reasonable that proteins may be involved in learning and memory. That increased protein synthesis could be one in a series of steps needed to form lasting or transient alterations in synaptic efficacy along specific neuronal pathways in neuronal remodelling is a reasonable hypothesis (Barondes 1965). Increased protein synthesis after training has been reported by many investigators using correlative studies, in which increases in RNA production (Hyden & Egyhazi 1964) and increases in the incorporation of labelled amino-acids into proteins have been found in specific brain regions of trained animals (reviewed by Roberts & Flexner 1969). More specifically, some of the proteins involved have been isolated;

Barraco & Irwin (1976) have reported that on training pigeons there is an alteration in four specific proteins isolated by gel electrophoresis, and Hyden & Lange (1970) have reported an increased synthesis of the protein S-100 in rats that have been trained on a reverse handedness task. Increases in protein synthesis in the chick brain have been found as early as 30 min after training (Mileusnic et al 1980) and have persisted (in the rat) for up to 8 hours (Hyden & Egyhazi 1964), while Zemp et al (1966) have found that incorporation of labelled amino-acids reverts to normal after 15 min in trained mice. There is thus a great deal of diversity in the literature as to the exact time at which protein synthesis de novo occurs, and for how long it persists after training.

An alternative to this hypothesis of protein synthesis de novo as the physiological mechanism underlying training, is the theory that memory formation takes place consequent to post- translational modification of existing proteins. The term post-translational modification includes the many known covalent modifications of proteins following their release from the ribosome. These include glycosylation-deglycosylation, sulphation-desulphation and phosphorylation-dephosphorylation processes (reviewed by Krebs & Beavo 1979). Research on protein sulphation mechanisms in general cell recognition and membrane modification processes is a currently active research area. The process of protein sulphation on tyrosine residues has been investigated in cells in culture and in-situ. Sulphation seems to be restricted to proteins of molecular weights in the range 120K - 80K, and these may have an important role in cell function, as they are found in most tissue cells (Huttner et al 1985). More specifically, there is evidence in the literature that does suggest that such modification of proteins is a direct consequence of training, in particular with regard to phosphorylation processes.

After training rats on a step-down avoidance task, Greengard (1978) and Routtenberg (1982) found that there was an increase in phosphorylation of a specific protein, F1 (mol wt 47 kDal), in the brains of trained over control animals. A more recent study by Routtenberg & Lovinger (1985) suggests that this increase in phosphorylation may be a consequence of increased electrical activity associated with the much investigated, training-related phenomenon of long term potentiation (LTP), as discussed in Chapter 2. Results from the experiments reported in this thesis and from previous *in vivo* studies (Sukumar *et al* 1980) indicate that there is an increase in fucose incorporation into particulate proteins in (M) trained over (W) control chicks. As fucose is a terminal sugar in glycoproteins (discussed in Chapter 2), it is conceivable that changes in fucose incorporation as a result of training may indeed be an example of a post-translational modification of pre-existing proteins. If this training-related increase in fucose incorporation is independent of protein synthesis *de novo*, then agents which selectively inhibit protein synthesis should have no effect on the increase in fucose incorporation.

The aim of the experiments reported in this Chapter were to investigate this hypothesis; to determine if the observed increase in fucose incorporation is a specific, induced, post-translational protein modification, or if it involves a dual process of initial increase in protein synthesis followed by an increase in fucosylation. This series of experiments investigated the effects of training on both protein synthesis and fucosylation *in vivo* and *in vitro* in chick forebrain and in chick forebrain slices, respectively.

5.1 MATERIALS AND METHODS

Training was carried out as previously described using Ross Chunky chicks 18 - 30 hr posthatch. Forebrains were removed and prepared exactly as described in Chapter 4.

Experiment 1

The first of this series of experiments was to determine the effect of 100 ul 30mM cycloheximide (CX) on fucose incorporation *in vitro*. Cycloheximide (Sigma), a protein synthesis inhibitor, was dissolved in deionised water and added to the incubation flasks before the addition of the tissue slices, at a final concentration of 1mM. At various intervals during the 3 hour incubation, approximately 3-4 slices (3-4 mg prot) were removed. These were homogenized and fucose incorporation into a trichloroacetetic acid (T.C.A.) insoluble portion analysed. Each flask containing cycloheximide was run in parallel with a control incubation (containing no cycloheximide), and slices from both flasks were removed at the same time.

Experiment 2

This investigation involved the analysis of the effect of 1 mM CX *in vitro* on fucose incorporation into slices of the right forebrain base of trained (M) and control (W) animals. Animals were trained and tested 30 min later. 15 min after testing, they were killed. The right forebrain base of 10 (M) trained and 10 (W) control animals was sliced and incubated as described. 100 ul 1mM CX was added to the incubation medium and after 3 hr fucose incorporation into a T.C.A. insoluble fraction was determined in slices from trained and control

chicks.

Experiment 3

The aim of this experiment was to determine the effect of CX *in vivo* on leucine incorporation *in vivo* and fucose incorporation *in vitro* in chick brain tissue.

10 min before training, either 2 x 10 ul 0.1mM CX dissolved in 0.9% saline (subsequently referred to as CX), or 2 x 10 ul saline (referred to as S), were injected freehand, intraventricularly into each chick hemisphere. The injections were made midway, anterio-posteriorly and approximately 1 - 2 mm from the midline. Cycloheximide and saline were injected using a Hamilton syringe fitted with a stopper to control the depth of injection to 3mm. All intracranial injections were performed by Steven Rose. 15 min after training, each chick was injected intraperitoneally with 50 MBq ^{3}H-leucine in 500 ul 0.15M saline, and 15 min after this they were tested. They were then left for a further 15 min before killing. Figure 5.1 schematically outlines the experimental procedure. This experiment involved 4 groups of chicks: (1) chicks injected with CX and trained on a methylanthranilate coated bead, CX(M), (2) chicks treated with CX and trained on a water coated bead, CX(W), (3) chicks treated with S and methylanthranilate trained, S(M) and (4) S treated and water trained, S(W). A total of 10 chicks were included in each group. The right base of each chick was subsequently removed, sliced and incubated in a medium containing ^{14}C-fucose. Three hours later, ^{14}C-fucose and ^{3}H-leucine incorporation into the homogenate and the T.C.A. insoluble portion of the right base samples were analysed. Radioactivity was counted using a dual label program on a Beckman LS 7500 scintillation counter to an efficiency of 76% for

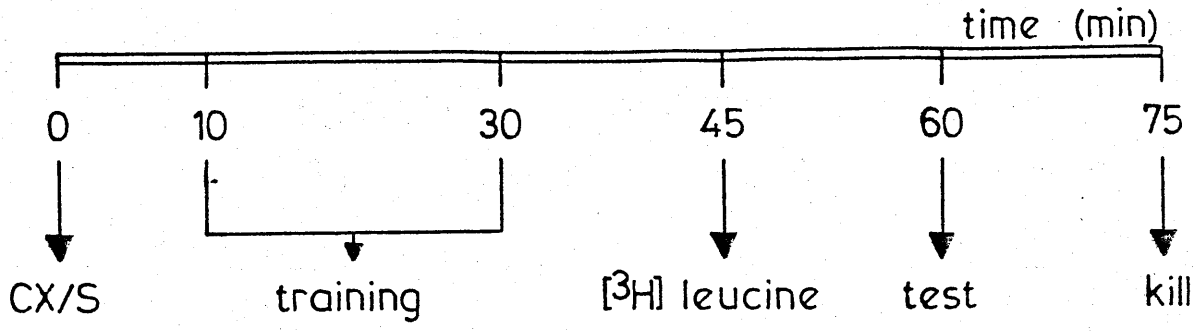


Figure 5.1. Summary of the experimental protocol employed in Experiment 3. Ten minutes before training, chicks were injected with either cycloheximide (CX) or saline (S). Fifteen minutes after training, they were injected with 50 MBq ^{3}H leucine, and 15 min later, they were tested. After a further 15 min period, the chicks were killed.

^{14}C and 41% for ^{3}H. The roof regions were retained and analysed for ^{3}H-leucine incorporation only. Each roof was homogenized in 4 ml 0.32 M sucrose using a Polytron (setting 4). 200 ul was removed for the assay of total homogenate radioactivity, and a further 200 ul was used to determine protein content by the method of Lowry et al (1951). The remainder was washed with 10% T.C.A. and leucine incorporation into the T.C.A. insoluble pellet measured.

Experiment 4

The purpose of this experiment was to determine the effect of an intraventricular injection of CX or S on leucine and fucose incorporation in vivo in chick brain after training.

CX or S injections were made as previously described, 10 min before training. 15 min after training, 500 ul 0.9% saline solution containing 20 MBq ^{14}C-leucine and 50 MBq ^{3}H-fucose was injected intraperitoneally into each chick. 15 min after this, the chicks were tested. They were then killed after a further 30 min. The training and testing protocol was identical to that used in the previous experiments, with the exception of a further 15 min delay before killing. This delay was to ensure that detectable quantities of labelled fucose would be incorporated. The experimental protocol is outlined in Figure 5.2.

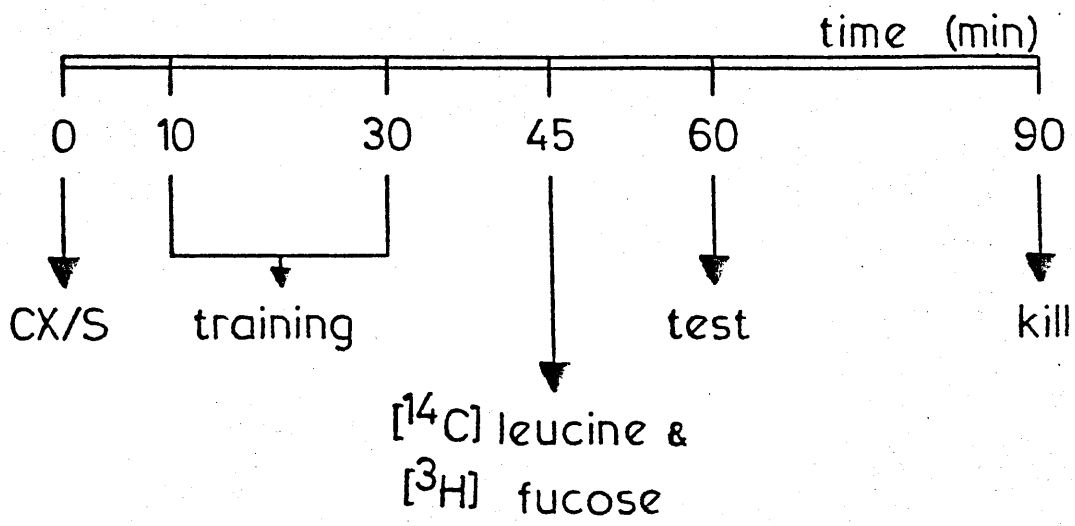


Figure 5.2. Summary of the experimental protocol employed in Experiment 4. Ten minutes before training, chicks were injected with either cycloheximide (CX) or saline (S). Fifteen minutes after training, they were injected intraperitoneally with 50 MBq ^{14}C leucine and 50 MBq ^{3}H fucose. They were tested 15 min later and, after a further 15 min period, the chicks were killed.

This experiment also involved four treatment groups (1) CX (M), (2) CX (W), (3) S (M) and (4) S (W), (these group definitions were similar to those described in experiment 3, with the exception that each chick

was also injected with ^{3}H-fucose 15 min after training). A total of 10 chicks were included in each treatment group. After killing, the brains were dissected into the four specified regions (RB, LB, RR and LR) and placed on ice. Each brain region was then homogenized in 4 ml 0.32M sucrose, and 4 x 200 ul removed for analysis of homogenate radioactivity and protein content (Lowry et al 1951). The remaining 3.2 ml was washed and centrifuged with 10% T.C.A. and the insoluble portion dissolved in protosol and added to 8 ml scintillation cocktail (Cocktail T BDH). Each sample was then counted for ^{14}C-leucine and ^{3}H-fucose incorporation, using a dual label program on a Beckman LS 7500 counter, with efficiences of 76% and 41% respectively.

Experiment 5

In this experiment, 100 ul samples of tunicamycin (Sigma), an antibiotic which inhibits glycosylation (Heifetz et al 1979), were added to the incubation medium containing chick forebrain slices, in concentrations ranging from 2.5 ug/ml - 1pg/ml. To monitor the effects of tunicamycin on both protein synthesis and fucosylation, 0.1mM leucine solution containing 3.7 MBq/ml ^{3}H-leucine was added to the medium, in addition to the normal 1mM fucose solution containing 3.7 MBq/ml ^{14}C-fucose. Each tunicamycin concentration was run in parallel with a control (containing no tunicamycin), and each concentration was studied in three separate experiments. After 3 hr, the slices were removed and homogenized, and leucine and fucose incorporation in the T.C.A. insoluble portion determined as described above.

Calculations

In any single experiment, all results were standardized around the grand mean of the entire experiment (a method of standarization which I have used throughout this thesis, and which reduces the variability inherent in working with chicks from different hatches). In experiments 1,2,3,and 5, fucose incorporation was calculated (as described in Chapter 4) in nmoles (L-^{14}C- fucose) incorporated /mg protein/hr, in which incorporation *in vitro* was investigated. In experiment 4, fucose incorporation was calculated as dpm/mg protein in the T.C.A. insoluble portion as a percentage of that found in the homogenate, (i.e. free + T.C.A. bound). This calculation was also used to express leucine incorporation values in all experiments in which incorporation *in vivo* was analysed. In experiment 5, leucine incorporation was calculated as nmoles/mg prot/ hr (*in vitro* analysis). The data from experiments 1 and 2 were statistically analysed using a Student's t-test (un-paired), while the data from experiments 3 and 4 were analysed using a nested design of a multivariate analysis of variance (MANOVA). This analysis was performed using the standard SPSS (MANOVA) package on the Cambridge computer.

5.2 RESULTS

Experiment 1

When 1mM cycloheximide was added to the incubation medium, there was a progressive inhibition of fucosylation up until 180 min. Figure 5.3 shows that a 60% inhibition of fucosylation compared with control values was recorded after 180 min of incubation. As inhibition was not 100%, this result indicated that after 3 hr, 40% of the recorded fucosylation was occurring on pre-synthesised (assuming that protein synthesis inhibition was 100%), unglycosylated protein. As a result of these observations, the next step was to determine if the observed increase in fucose incorporation after training in slices from (M) trained chicks compared to (W) controls still persisted when cycloheximide was added to the incubation medium.

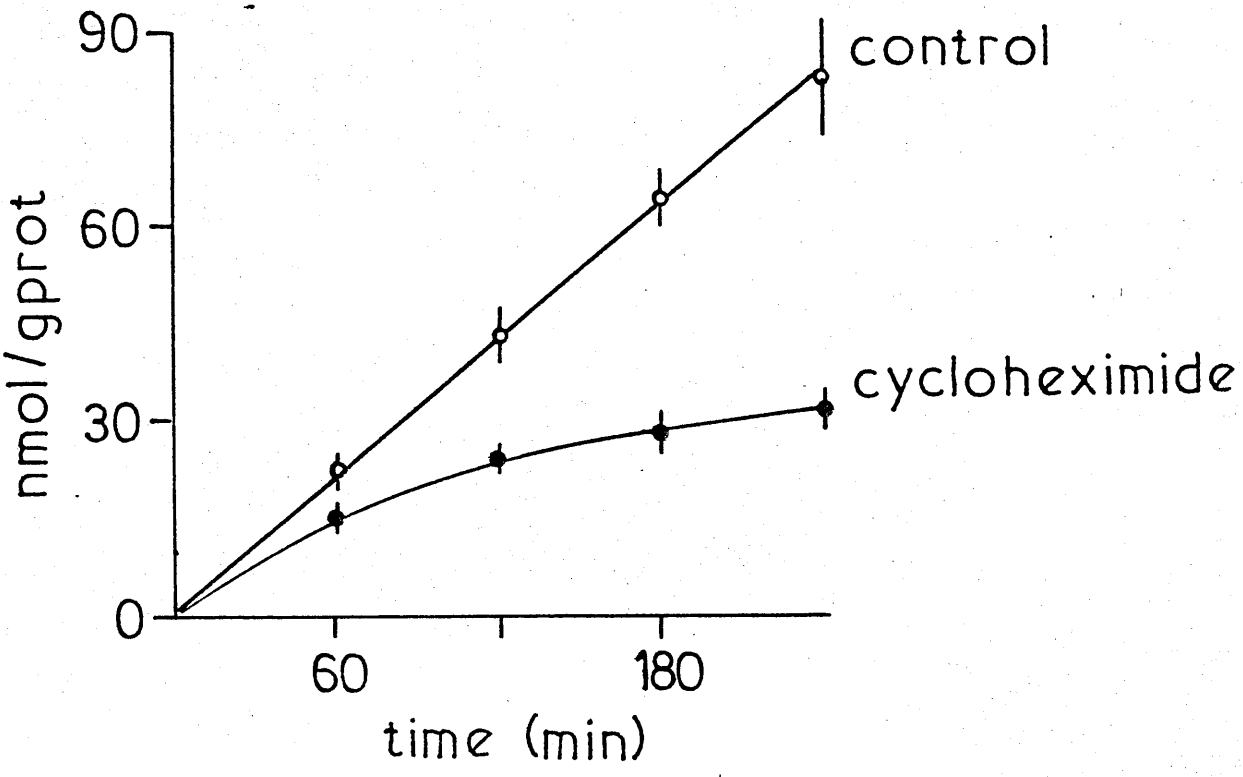


Figure 5.3. Forebrain slices were prepared and incubated as described in Materials and Methods. In one set of incubations, 1mM cycloheximide was added. Incubation was stopped at various times (up to 3 hr); fucose incorporation into an acid-insoluble fraction was then determined in the control and CX slices. Values represent mean ± SEM of three separate determinations.

Experiment 2

Experiment 2 involved the addition of 1 mM cycloheximide to the incubation media containing slices of the right forebrain base regions of trained and control chicks. A 65% inhibition of absolute fucosylation values was recorded when fucose incorporation in CX treated slices was compared to that found in untreated slices. However, as Table 5.1 shows (no Cx), the significant increase in fucose incorporation of 16% ($P<0.05$), previously observed *in vitro* in trained animals over controls (see Chapter 4), still persisted. A significant increase of 19% ($P<0.05$) in fucose incorporation was still evident in (M) trained chicks over (W) controls (CX in medium). This result seemed to favour the hypothesis that the increase in fucosylation, as a result of training, was occurring independently of protein synthesis *de novo*. However, one cannot completely accept this hypothesis, as a training-related increase in protein synthesis may have been established in the 45 min period between training and the addition of the slices to the medium containing cycloheximide. If this increase in protein synthesis did occur *in vivo*, then these proteins may have been the substrates for the observed increase in fucosylation *in vitro*. In order to block this *in vivo* protein synthesis , I decided to inject CX or S intracranially before training. To ensure that inhibition of protein synthesis was occurring, each chick was injected intraperitoneally 15 min after training with labelled leucine.

Table 5.1

The Effect of 1mM CX on Fucose Incorporation *in vitro*

Region	nmoles/g protein/ hr (M) trained	(W) control	M/W x 100
CX in medium			
Right Base	12.84 ± 0.44	10.76 ± 0.26	119*
No CX			
Right Base	36.84 ± 1.34	31.57 ± 1.11	116*

Chicks were trained and slices from the right forebrain base were prepared as described in Materials and Methods. In one set of incubations, 1 mM cycloheximide (CX) was added. Fucose incorporation *in vitro* in an acid-insoluble fraction of slices incubated in the presence and absence of CX was determined after 3 hr. Values represent mean ± SEM of 10 separate determinations.
*$P < 0.05$ (Student's t-test, two-tailed).

Experiment 3

Experiment 3 was thus concerned with the intraventricular injection of CX or S in trained and control chicks 10 min before training, followed by an interperitoneal injection of labelled leucine 15 min after training.

In order to ascertain the behavioural effects of the intraventricular injections, data on the number of chicks reaching training criteria in both the (M) trained and (W) control groups was expressed as a percentage of the initial number of chicks in each group, and comparisons were made with percentage values obtained from untreated, trained chicks. (Chicks that met training criterion in the (M) trained group were chicks that had pecked and avoided the methylanthranilate coated bead and on representation of a dry bead had avoided it. In the (W) control group, chicks that had pecked a water coated bead and had subsequently pecked on a second presentation of a dry bead were accepted as meeting the training criterion). Table 5.2 shows that the percentage of birds treated with saline, whether (W) controls or (M) trained, that reached training criteria were similar to those recorded in previous training experiments (see Chapter 3), when chicks were untreated. In the untreated group the percentage of chicks reaching training criterion was 94% (W) control and 77% (M) trained compared to 80% (W) control and 75% (M) trained for the saline treated group. However in the cycloheximide group, the number of (M) trained chicks attaining criterion was reduced, 41% being recorded against 75%, in the untreated (M) trained group. However, 70% of the total (W) control chicks treated with CX reached criterion which was similar to 80% found in the untreated (W) control group.

Table 5.2

The Percentage of Chicks which reached Training Criteria

TREATMENT	(M)trained	(W)controls
CYCLOHEXIMIDE	41%	70%
SALINE	77%	94%
UNTREATED	75%	80%

Three groups of chicks were taken. One group was injected intraventricularly with 1mM cycloheximide 10 min before training, the second was injected with saline and the third was left untreated. The number of chicks reaching training criteria ((M) trained and (W) control) was calculated as a percentage of the total number of chicks trained in each of the three groups. The total number of chicks trained in each group was 40 (20 (M) trained and 20 (W) control).

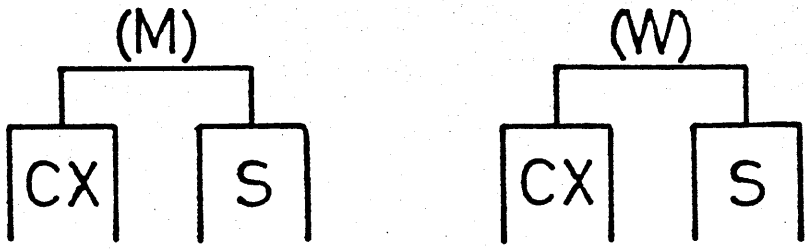
Each chick was injected with ^{3}H-leucine to monitor the effects of protein synthesis inhibition due to cycloheximide *in vivo*. Analysis of the data was performed using a Multivariate Analysis of Variance (MANOVA), such that two sets of data was obtained; (1) the effect due to treatment (CX and S), within training was determined (i.e. incorporation of leucine and fucose in the CX (M) trained compared to that in the S (W) control and incorporation in the S (M) trained compared to that in the S (W) control) and (2) the effect of training within treatment (i.e. incorporation in the CX (M) trained compared that in the CX (W) control and incorporation in the S (M) trained compared to that in the S (W) control.

Table 5.3 shows the data for leucine incorporation *in vivo* in the roof regions. Table 5.3 A shows that there is a significant decrease in leucine incorporation due to CX treatment in both the (M) trained and (W) control chicks. There is a significant inhibition in leucine incorporation of 60% ($P<0.001$) and of 59% ($P<0.001$) in the CX (M) trained compared to the S (M) trained and in the CX (W) controls compared to the S (W) controls, respectively. Table 5.3 B shows the effect of training within treatments, and indicates that there is no effect due to training when leucine incorporation in the CX (M) trained chicks is compared to that in the CX (W) controls, and no difference in incorporation is found in the S (M) trained chicks compared to S (W) controls ($P>0.05$) MANOVA.

Table 5.3

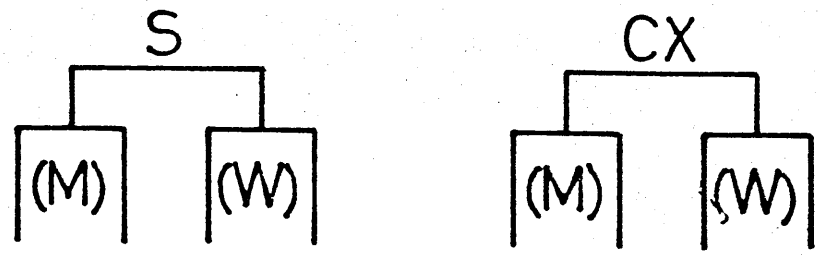
Leucine incorporation in-vivo in the roof regions.

(a) Treatment within training.



Region	CX(M) % difference	S(M) P value	CX(W) % difference	S(W) P value
Roof	-60	0.000*	-59.4	0.000*

(b) Training within treatment



Region	S(M) % difference	S(W) P value	CX(M) % difference	CX(W) P value
Roof	1.6	0.864	0.7	0.972

Table 5.3. Chicks were injected with either CX or S 10 min before training. Fifteen minutes after training, they were injected with ^{3}H leucine. The chicks were then killed, the forebrain roof obtained and leucine incorporation into an acid-insoluble fraction determined. In (a), the results of the effect of 'treatment within training' (as described in the text) on incorporation are shown. The diagram indicates which groups were statistically compared. In (b), the effect of 'training within treatment' on incorporation is shown.

The percentage difference between each compared group is given in the Table: + indicates an increase, - indicates a decrease. P values were obtained from a multivariate analysis of variance (MANOVA).

n = 10 in each group.

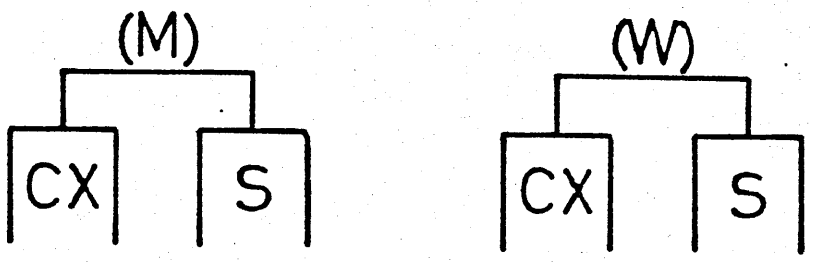
* $P < 0.001$.

Table 5.4 shows the data for leucine incorporation *in vivo* in the right base. Table 5.4 A shows the effect of CX treatment compared to S in both the (M) trained and (W) control chicks. There is a significant inhibition in leucine incorporation of 58% ($P<0.001$) in the (M) trained chicks due to CX treatment when incorporation in the S (M) trained are compared to the incorporation in the CX (M) trained chicks. A significant inhibition in leucine incorporation of 46% ($P<0.001$) is also evident due to CX treatment in the CX (W) controls compared to that in the S (W) control chicks. Table 5.4 B shows the effect of training within treatment (CX and S), and indicates that there is no difference in leucine incorporation due to training when comparisons are made between leucine incorporation in the S (M) trained and S (W) controls ($P>0.05$) and between CX (M) trained and CX (W) controls ($P>0.05$).

Table 5.4

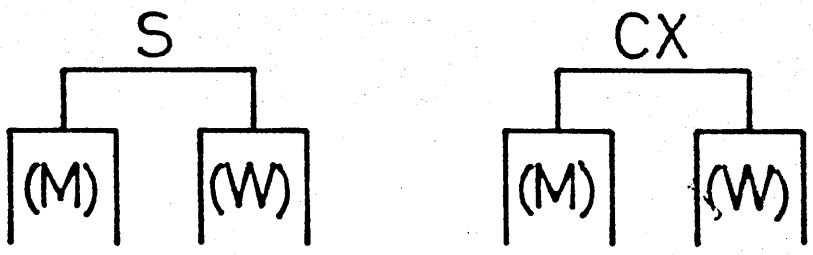
Leucine incorporation *in vivo* in the right base.

(a) Treatment within training



Region	CX(M) % difference	S(M) P value	CX(W) % difference	S(W) P value
Right Base	-57.8	0.000*	-45.5	0.000*

(b) Training within treatment



Region	S(M) % difference	S(W) P value	CX(M) % difference	CX(W) P value
Right Base	6.8	0.297	-20.2	0.297

Table 5.4. Chicks were injected with either CX or S before training. Fifteen minutes after training, they were injected intraperitoneally with ^{3}H leucine. The chicks were then killed, and the right forebrain base was sliced and incubated in a medium containing ^{14}C fucose. After 3 hr, leucine incorporation (*in vivo*) in an acid-insoluble fraction of right forebrain base was determined. In (a), the effect of 'treatment within training' is shown; in (b), the effect of 'training within treatment' is shown.

Data represent the percentage difference between the compared groups; P values were obtained from a MANOVA.

n = 10 in each group.

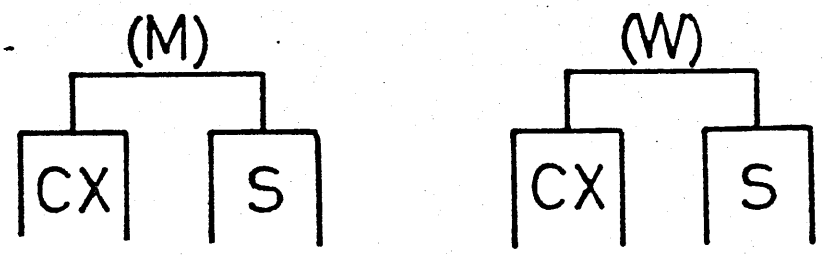
* $P < 0.001$.

Table 5.5 shows the statistical analysis of fucose incorporation rates in the right forebrain slices *in vitro*. Table 5.5 A shows the effect of CX on fucose incorporation rates when compared to S treatment. There was no significant difference in fucose incorporation *in vitro* due to CX treatment *in vivo*, when incorporation rates were compared in the S (M) trained chicks against the CX (M) trained ($P>0.05$), nor was there any difference in fucose incorporation between the S (W) controls and the CX (W) controls ($P>0.05$). On analysis of training within treatments, there was a significant increase of 16.8% ($P<0.05$) in fucose incorporation in the CX (M) trained chicks compared to the CX (W) controls. However, no such training-related increase in fucose incorporation was evident in the S (M) trained chicks compared to the S (W) controls ($P>0.05$) MANOVA.

Table 5.5

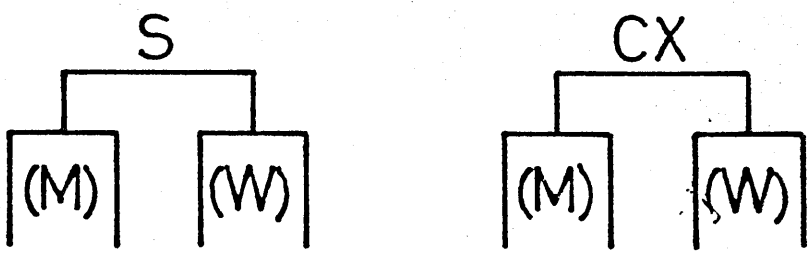
Fucose incorporation *in vitro* in the right base

(a) Treatment within training



	CX(M)	S(M)	S(W)	CX(W)
Region	% difference	P value	% difference	P value
Right Base	-4.97	0.572	11.19	0.157

(b) Training within treatment



	S(M)	S(W)	CX(M)	CX(W)
Region	% difference	P value	% difference	P value
Right Base	1.67	0.840	16.8	0.032*

Table 5.5. Chicks were injected with either CX or S before training. Fifteen minutes after training, they were injected intraperitoneally with ^{3}H leucine. The chicks were then killed and right forebrain bases were incubated in a medium containing ^{14}C fucose. After 3 hr, fucose incorporation *in vitro* in an acid-insoluble fraction was determined. In (a), the effect of 'treatment within training' is shown; in (b), the effect of 'training within treatment' is shown.

Data represent the percentage difference between the compared groups; P values were obtained from a MANOVA.

n = 10 in each group.

* $P < 0.05$.

The results from this experiment indicated that after an intraventricular injection of CX, protein synthesis *in vivo* was significantly inhibited by 60% in the roof region and 50% in the base region. However, CX *in vivo* had no effect on fucose incorporation rates *in vitro* in right forebrain base slices. On statistical analysis of fucose incorporation rates *in vitro* in CX (M) trained compared to incorporation in S (M) trained and between CX (W) controls and S (W) controls, there was no difference ($P>0.05$). However, when the effects of training within treatments was analysed, there was a significant increase in fucose incorporation rate *in vitro* in the CX (M) chicks compared to the CX (W) controls ($P<0.05$). Such a training-related increase was not found in the S (M) trained chicks compared the S (W) controls ($P>0.05$).

There are two major difficulties with the interpretation of this data: (1) protein synthesis was only inhibited by 50% *in vivo*, and there was a possibility that the CX effect was washed out durning the 3 hr incubation period, such that protein synthesis *de novo* was occurring, and (2) although there was a significant increase in fucose incorporation in CX (M) trained chicks over the CX (W) controls *in vitro* in forebrain base slices, no such increase was found in the S (M) trained chicks when compared to the S (W) controls in *in vitro* (which was the essential 'sham control' group). To investigate further the involvement of protein synthesis *de novo* in the observed training related increase in fucose incorporation, a different experimental design was devised. In the following experiment, I decided to look simultaneously at the effects of training on protein synthesis and fucosylation *in vivo* in CX and S treated chicks.

Experiment 4

In experiment 4, CX or S was injected intraventricularly, and ^{14}C-leucine and ^{3}H-fucose intraperitoneally in (M)trained and (W) control chicks. Thirty minutes after testing, leucine and fucose incorporation in vivo was determined in the four brain regions. This data was analysed using a nested design of a Multivariate Analysis of Variance (MANOVA). I wanted to obtain two distinct sets of information from these data as in experiment 3: (1) overall effect of CX and S treatments on incorporation values: treatment within training. In order to determine this, the incorporation of both leucine and fucose was compared in CX (M) controls against that in S (M) trained and incorporation in CX (W) controls was compared against that in S (W) trained, such that the only variable was treatment (either CX or S). These values were obtained for each region. Subsequently, the data was then analysed to determine the second set of information; (2), the effect of training on incorporation values within treatments (CX and S); training within treatment. In each region, incorporation of both labels was compared, S (M) trained against S (W) controls and CX (M) trained against CX (W) controls; in this analysis, the variable was training (M) against (W). The former analysis thus gives the results of treatment effects within training, while the latter gives the results of training effects within treatments, for each brain region (thus I used a nested design of MANOVA). Table 5.6 shows the statistical results for leucine incorporation in all four brain regions. Table 5.6 A shows the effects of treatment (CX and S) on leucine incorporation. There is a significant difference in leucine incorporation in the saline treatment group compared to the CX treated group. There is a significant inhibition of 30% ($P<0.025$) in leucine incorporation due to CX treatment between the CX (M) trained and S (M) trained and also

between CX (W) controls and S (W) controls. This significant inhibition is found in all four forebrain regions. Table 5.6 B shows the data on the effect of training within treatments and indicates that within the S group there is a significant decrease of 18% ($P<0.025$) in leucine incorporation in the left roof of (M) trained chicks compared to (W) controls. In the CX treated group, there is a significant decrease in leucine incorporation in the (M) trained chicks compared to the (W) controls in three regions; right base of 24% ($P<0.05$), right roof of 21% ($P<0.005$) and left roof of 19% ($P<0.001$).

Table 5.6

Leucine incorporation in vivo

(a) Treatment within training

	CX(M)	S(M)	CX(W)	S(W)
Region	% difference	P value	% difference	P value
Right Base	-34.3	0.014*	-23.7	0.001*
Left Base	-37.8	0.008	-22.5	0.000*
Right Roof	-30.1	0.002	-26.2	0.008*
Left Roof	-36.3	0.000*	-35.6	0.000*

(b) Training within treatment

	S(M)	S(W)	CX(M)	CX(W)
Region	% difference	P value	% difference	P value
Right Base	-12.7	0.212	-31.9	0.037*
Left Base	-0.05	0.701	-28.4	0.057*
Right Roof	-18.1	0.103	-26.0	0.037*
Left Roof	-21.0	0.012*	-22.4	0.000*

Table 5.6. Ten minutes before training, chicks were injected with either CX or S. fifteen minutes after training, they were injected intraperitoneally with ^{14}C leucine and ^{3}H fucose. After 30 min, the chicks were killed and leucine and fucose incorporation into an acid-insoluble fraction determined for four regions of forebrain. In (a), the effect of 'treatment within training' on leucine incorporation is shown; in (b), the effect of 'training within treatment' is shown.

Data represent the percentage difference between the compared groups; P values were obtained from a MANOVA.

n = 10 in each group.

* $P < 0.05$.

Table 5.7 shows the analysis of fucose incorporation due to CX and S treatments and training *in vivo*. Table 5.7 A shows the effect of treatment on fucose incorporation in the four brain regions. There is a significant inhibition in fucose incorporation due to CX treatment over S treatment in CX (M) trained chicks compared to S (M) trained of 76% ($P<0.001$), and between the CX (W) controls and S (W) controls of 35% ($P<0.05$). This indicates that CX caused a significant inhibition in fucose incorporation *in vivo* in all brain regions in both the (M) trained and (W) control chicks. Table 5.7 B shows the effect of training within treatments. There is a significant increase of 40% ($P<0.025$) in fucose incorporation due to training in the S (M) trained chicks compared to that in the S (W) controls in all four brain regions. However, within the CX treatment group there was no difference in fucose incorporation due to training in any region of the CX (M) trained chicks compared to the CX (W) controls ($P>0.05$).

Table 5.7

Fucose incorporation *in vivo*

(a) Treatment within training

	CX(M)	S(M)	CX(W)	S(W)
Region	%difference	P value	% difference	P value
Right Base	-76	0.000*	-35.7	0.039*
Left Base	-84	0.000*	-48.6	0.035*
Right Base	-76.4	0.000*	-52.1	0.017*
Left Roof	-76.3	0.000*	-56.1	0.016*

(b) Training within treatment

	S(M)	S(W)	CX(M)	CX(W)
Region	% difference	P value	% difference	P value
Right Base	45.6	0.007*	32.2	0.435
Left Base	42.6	0.001*	25.5	0.563
Right Roof	41.1	0.024*	16.4	0.718
Left Roof	38.1	0.009*	12.6	0.810

Table 5.7. This Table presents data on fucose incorporation *in vivo* in four regions of the chick forebrain. The experimental protocol was exactly the same as that given in the legend to Table 5.6. In (a), the results of the effect of 'treatment within training' on incorporation are shown. In (b), the effect of 'training within treatment' is shown.

Data represent the percentage difference between the compared groups; P values were obtained from a MANOVA.

n = 10 in each group.

* $P < 0.05$.

These results indicate that when protein synthesis is significantly inhibited *in vivo* by 50%, there was a significant inhibition of fucose incorporation *in vivo*, as opposed to the effects on fucose incorporation *in vitro* (Experiment 3). There was a significant increase in fucose incorporation in S (M) trained over S (W) control chicks in all four brain regions. However, in the CX treated birds, there is no training related increase in fucose incorporation in any brain region of the CX (M) trained chicks compared to the CX (W) controls. That a training related increase in fucose incorporation was found in the chicks treated with saline and no increase was evident in those birds treated with a protein synthesis inhibitor, suggests that the observed increase in fucose incorporation was dependent on protein synthesis *de novo*. However, one cannnot rule out the possibility that CX directly inhibits fucose incorporation into proteins by inhibiting intracellular mechanisms, as this has not been investigated. However, Cx does not seem to be having any effect on fucose uptake as analysis of intracellular fucose levels in the Cx treated chicks is not significantly different to that in the S treated chicks.

Experiment 5

An investigation to determine the effect of a range of tunicamycin concentrations on fucose incorporation and leucine incorporation *in vitro* was conducted. Table 5.8 shows that, with the highest tunicamycin concentration used (100 ug/ml) 64% of the control fucose incorporation rate (slices incubated with no tunicamycin) was detected, indicating a 36% inhibition. When the lowest concentration (0.01 g/ml) was added to the incubation medium, fucose incorporation was 87% that found in slices in the control incubations. On analysis of leucine incorporation in slices in control and tunicamycin

containing incubations, a similar trend was found. With increasing tunicamycin concentrations, there was an increasing inhibition of leucine incorporation. These experiments indicate that with increasing concentrations of tunicamycin, there was an increasing inhibition of fucosylation, but that coupled with this, there was a non-specific inhibition of protein synthesis *in vitro*.

Table 5.8

The Effect Of Tunicamycin on Fucose and Leucine Incorporation In Chick Forebrain Slices.

Tunicamycin Conc ug/ml	^{14}C-Fucose	^{3}H-leucine
	% Incorporation of Control	
1000	64	66
500	62	68
125	50	85
2.5	64	73
0.01	87	89

Slices from chick forebrain were prepared and incubated as described in Materials and Methods. Included in the medium was 3.7 MBq/ml ^{3}H leucine and 3.7 MBq/ml ^{14}C fucose, with a range of concentrations of tunicamycin (1000 ug/ml to 0.01 ug/ml). After 3 hr incubation, the incorporation of leucine and fucose into an acid-insoluble fraction was determined. Each tunicamycin concentration was run in parallel with a control incubation (no tunicamycin). Values represent the incorporation rate of leucine and fucose as a percentage of incorporation in a control incubation, and are the means for three separate determinations.

5.3 DISCUSSION

When given shortly before or after training, protein synthesis inhibitors block memory formation, as seen when the animal is tested at specific times after training; amnesia for the task is evident (Davis & Squire 1984). Cycloheximide, acetoxycycloheximide and puromycin are the three most studied protein synthesis inhibitors. Mark & Watts (1971) found that CX injected intracerebrally shortly before or up to ten minutes after training, in doses of 37.5 ug/chick, caused a gradual loss of retention for a passive avoidance task up to three hours after training, with 80% of the animals showing amnesia. Barondes & Cohen (1966) showed that mice can be trained in the presence of puromycin which inhibited cerebral protein synthesis by more than 80%. Memory for the task declined following learning, until maximum loss of recall was recorded after three hours. With acetoxycycloheximide (Cohen & Barondes 1968a) and cycloheximide (Cohen & Barondes 1968b), memory persisted for at least three hours after training but was completely lost six hours later. Agranoff & Davis (1967) have shown memory to persist for six hours in goldfish under the influence of puromycin and to decay after this period. Thus, there is a lot of confusion from such diverse results as to the involvement of protein synthesis in memory formation, and more specifically, the time after training at which protein synthesis de-novo is required. This research has been conducted using rodents, fish, and birds and has employed a variety of training tasks and specific protein synthesis inhibitors (reviewed by Agranoff et al 1978). This form of interventive research attempts to correlate inhibition of protein synthesis and RNA synthesis with behavioural and/or biochemical effects. Originally, the principal appeal of these interventive agents was based on the assumption that they permit the block of discrete, synthetic processes in the intact behaving animal,

with little direct effect on overall metabolism. More recently however, this area of research has attracted critics, mainly due to the uncertainty attached to unspecific action and unspecific sites of action of the inhibitor used. A perfect example of this is the amnestic agent-puromycin, which has been extensively used in this area of research. It is now known to have so many non-specific effects (including diminished and irregular electrical activity which leads to a convulsive state, as observed in mice) that it is now rarely used (Dunn 1980). Indeed, an excellent review by Roberts & Flexner (1969) on the specificity, or more accurately, the lack of specificity, of protein synthesis inhibitors indicates that one must excercise extreme care in choosing one to work with. As a result, one must also consider possible nonspecific actions when drawing experimental conclusions.

In this set of experiments, I wanted to determine the involvement of protein synthesis de-novo (if the observed increase in fucose incorporation observed 30 min after training in vitro was occurring on de novo protein synthesis), or if the observed increase was an independent biochemical effect occurring on proteins already present. In order to investigate this problem, I used the protein synthesis inhibitor cycloheximide (CX) both in vivo and in vitro. The training-test period was 30 min, as in previous experiments in which increased fucose incorporation was detected. Although a percentage of the CX treated chicks were amnestic, all chicks used for experimental analysis did reach training criteria. I was interested in the biochemical effects of CX as opposed to the behavioural effects, in particular in relation to the significant increase in fucose incorporation observed after training.

Cycloheximide, along with the closely related acetoxycycloheximide, belong to a class of antibiotics known as glutarimides. Their mode of action is by inhibition of peptide chain initiation as well as chain elongation, by interaction with the large 60s ribosomal subunits. Glutarimides interfere with several steps involved in the translocation of the peptide chain the ribosomes, including the release of messenger RNA along the ribosome (Flood et al 1972).

When 1mM cycloheximide (at a concentration which inhibited protein synthesis by 95% in chick forebrain slices in vitro: see Schliebs et al 1985) was added to the incubation medium, inhibition of fucosylation was progressive until three hours. After three hours an inhibition of 60% was observed in the slices treated with CX compared with slices incubated in the absence of CX. This initially suggested that there was some fucosylation taking place on protein precursors already present. This observation then stimulated the idea that, perhaps, the increased fucose incorporation observed after training chicks on P.A.L. was indeed solely due to increased fucosylation independent of de novo protein synthesis. To test this hypothesis, I trained chicks (as previously documented) and placed slices of the right forebrain base (the region in which the increase was detected), of (M) trained and (W) control chicks in an incubation medium containing 1 mM CX. After 3 hr incubation, a significant increase in fucosylation was detected in the (M) trained chicks compared to that in the (W) controls, although absolute fucosylation values were reduced by some 60%. However, assuming that protein synthesis in vitro was inhibited by some 95% by the addition of CX, one could not be sure that some protein synthesis was not occurring in vivo during the training and testing periods (45 min in total). If this was the case, then the observed increase in fucose incorporation in vitro

could have occurred on protein synthesized de novo during this time.

An experiment was thus designed such that this protein synthesis in vivo was inhibited by the intraventricular injection of CX. The concentration of CX used (0.07 nM, assuming the chick forebrain occupies a volume of 1 ml) and route of injection were similar to those used by Gibbs et al (1973), in which it was found that there was an 80% inhibition of protein synthesis in a post-mitochondrial fraction. The effect of cycloheximide on memory retention for particular tasks has been well studied in a variety of species. It was more effective if given before training (in which case training occurred during maximum inhibition of cerebral protein synthesis), and had a relatively slight, but significant effect if given subcutaneously immediately after training but none at all 30 min later (Agranoff et al 1967).

In all of the experiments in which CX was injected intraventricularly, all the chicks (both (M) trained and (W) controls) used for biochemical analysis met training criteria. However it was evident that the CX treated chicks were more disinterested and showed signs of lethargy when compared to their saline treated partners. The behavioural data (Table 5.2) show that there was a reduction in the percentage of chicks treated with CX and (M) trained that reached criterion compared to the percentage of chicks in the untreated (M) trained and in the Saline (M) trained group. This was probably due to the amnestic effect of CX, which is evident as early as 30 minutes after training; this was the training-test time used in these experiments. Gibbs & Ng (1979) found that when CX is injected intraventricularly 10 minutes before training chicks on a passive avoidance task, retention for the task begins to decline after 30 minutes following training with significant decreases in the

percentage of birds showing retention after 60 minutes. There was no difference in the percentage of (W) control chicks that reached training criteria in any of the treatment groups. That one observes overall behavioural effects due to CX treatment is hardly surprising, considering the many pharmacological effects protein synthesis inhibitors have, as discussed earlier. Cycloheximide has been shown to cause disruption of cerebral metabolism, including inhibition of catecholamine synthesis from tyrosine, thus increasing tyrosine levels (Flexner & Goodman 1975), However, Spanis & Squire (1978) have performed experiments which show that the amnestic effect of cycloheximide cannot be attributed to this elevation. A study by Hambley & Rogers (1979) showed that the effect of learning retardation in chicks which have been treated with an intraventricular injection of CX may be attributed to the accumulation of intracellular amino acids. In particular, they found an increase in the intracellular concentration of two putative excitatory transmitters, glutamate and aspartate. More specifically, Gibbs et al (1977) found that an excess of a transportable, but not a metabolizable amino-acid, -aminoisobutyrate, causes a CX-like amnesia in day-old chicks trained on a passive avoidance paradigm. However, every protein synthesis inhibitor has its disadvantages, and I decided to use CX as it is the one with which most chick behavioural studies have been performed (Mark & Watts 1977).

The results indicate that there was a significant inhibition (50%) in protein synthesis in vivo due to the CX treatment when compared to the S treatment in the two forebrain regions analysed, the right base and the roof region. There was no difference in leucine incorporation in the roof region due to training in the saline treated group, which is contrary to the results of Mileusnic et al (1980). They found a significant increase in leucine incorporation in the roof

of trained over control chicks 30 min after training. In the roof region, a pulse time of 30 minutes was used for the labelled leucine, which is the same as that used by Mileusnic et al (1980). However, analysis of leucine incorporation in my experiments was not performed immediately; instead, the tissue was stored at 4 C for up to 36 hours. This may have led to some proteolysis and thus distortion of results. On analysis of leucine incorporation in the right base regions of S (M) trained and S (W) controls, no difference in incorporation due to training was detected. This may have been due to the radioactive pulse time of leucine used in this experiment which was 3.5 hours, (0.5 hr in vivo and 3.0 hr in vitro). At this pulse time, leucine incorporation would have reached plateau levels, such that changes in incorporation would be undetectable. However, analysing a training related increase in leucine incorporation was not my main concern; rather, I was interested in determining the effect of an intraventricular injection of CX on protein synthesis in vivo, which was a significant 50% inhibition.

There was no effect of the intraventricular injection of CX on the absolute values of fucosylation in vitro in the right forebrain base slices, compared to fucosylation rates recorded from slices of S treated chicks. There was a significant increase in fucosylation in CX (M) treated birds over CX (W) chicks, but no increase was observed in the S (M) over the S (W) chicks. That there was an increase in the CX treated (M) chicks indicated very strongly that increased fucosylation may be occurring independent of protein synthesis as a result of training, but there were several reservations with this conclusion. First, protein synthesis was only inhibited by 50% in vivo while fucosylation in vitro was unaffected by the intraventricular injection of CX. This suggested that perhaps the observed increase in fucose incorporation was occurring on those 50%

proteins whose synthesis was not inhibited, or indeed that the CX effect was washed out during the 3 hr incubation such that protein synthesis de novo was restored. Secondly, no increase in fucosylation was observed in the S treated (M) chicks over S (W) controls. This treatment group was acting as a 'sham control' group for the CX treated group, and as such one could not draw conclusive results from this experiment. To solve the former problem I conceived two options, either to increase the dose of CX to increase the inhibition of protein synthesis or to use a different inhibitor. Unfortunately, I could not increase the dose of CX, as the working dose was near lethal concentrations and was having a marked debilitating effect on general behaviour. The alternative was to work with a different protein synthesis inhibitor, anisomycin. Bennett et al (1972) showed that anisomycin is an effective amnestic agent in rats and mice, and that it lacks some of the complications of cycloheximide. It is much less toxic than other protein synthesis inhibitors, and in mice, 25 times the effective amnestic dose was not lethal, whereas cycloheximide had to be used at doses near to the lethal dose to prevent memory formation (Rosenzweig 1984; Flood & Jarvik 1976). However, I decided not to work with anisomycin as I did not wish to introduce a new inhibitory agent and thus a new variable into my experiments at this stage, as I could not control for nor assess its side-effects.

The latter problem, of no increase in fucose incorporation in the S treated group as anticipated, was unexpected, and can only be explained by the uncontrolable, intrinsic variability in the chick's behaviour. I have discussed the inability to replicate results in biochemical/ behavioural experiments in Chapter 2, and this is an example of the difficulties associated with such work.

That an intraventricular injection of CX caused a 50% inhibition in protein synthesis *in vivo* and no inhibition of fucosylation *in vitro* may indicate that the observed increase in fucosylation was occurring on protein synthesis *de novo* (which was unaffected by CX). Therefore, it was still unclear as to the involvement of protein synthesis *de novo* in the observed biochemical effect detected after training.

Consequently, an experiment was conducted in order to simultaneously investigate the effects of intraventricular injections of CX or S *in vivo* on leucine and fucose incorporation *in vivo*, after training. This experimental protocol was undertaken such that a more accurate analysis of protein synthesis and fucosylation could be established. The data showed that CX caused a significant inhibition of 30% in protein synthesis *in vivo* over that recorded for S treated chicks in all brain regions. When the training effect within treatments (CX and S) was analysed, there was a significant increase in leucine incorporation in left roof of S (W) control compared to that in the S (M) trained chicks. Within the CX treated group there was a significant increase in leucine incorporation in all four brain regions in the (W) control chick compared to the (M) trained. That significant increases in leucine incorporation were found in regions of the (W) control birds compared to the (M) trained was unexpected and contrary to those results of Mileusnic *et al* (1980), which indicated an increase in leucine incorporation as a result of training in the forebrain roof of (M) trained chicks compared to (W) controls. That an increase in leucine incorporation was found in the S (W) control compared to the S (M) trained chicks as well as the increase found in the CX treated group, would tend to rule out the possibility that the significant differences in incorporation were due to the effect of CX treatment alone, especially in relation to the reported

effect of CX on the accumulation of intracellular amino-acids (Hambley & Rogers 1979). Perhaps these differences in incorporation values represent initial differences in intracellular pool-sizes of amino-acids. The fact that protein synthesis was inhibited by 30% in this experiment compares with the inhibition value of 50% recorded in the previous experiment (where there was no difference in leucine incorporation values in the S (W) compared to that in the S (M) trained chicks, and also no increase in incorporation was found in the CX (W) controls when compared to that in the CX (M) trained).

There was a significant inhibition in fucose incorporation in vivo in all brain regions due to CX, and this was apparent in both the CX (M) trained compared to that in the S (M) trained and also in the CX (W) controls compared to incorporation in the S (W) controls. There was no difference in fucose incorporation due to training in any region of the CX (M) trained compared to that in the CX (W) control chicks. However, in the S treated chicks there was a significant incorporation of fucose in the (M) chicks over the (W) controls and this increase was found in all brain regions. These data indicate that the fucosylation in vivo observed in S treated (M) trained chicks over (W) controls is abolished when CX is injected intraventricularly before training. This would indicate that when protein synthesis is inhibited in vivo before training (in this experiment by 30%), the training-related increase in fucose incorporation in vivo is also inhibited. This suggests that the observed increase in fucose incorporation after training is occurring on protein synthesis de-novo.

That the increase in fucosylation was located in the right base compares well with previous in vitro studies (described in Chapter 4) and with the in vivo study of fucose incorporation reported by Rose &

Harding (1984). Increases in fucokinase activity reported by Lossner & Rose (1984) were also located to the right forebrain base. The increase in fucose incorporation found in the left base of trained chicks in this in vivo study may be related to the increase in fucokinase activity found 1 hour after training as reported in Chapter 3, and may be further evidence for the involvement of the whole base region in biochemical changes correlated with training. Furthermore, the increase in fucose incorporation in vivo in the right and left roof regions reported in this study is the first time that a training related biochemical change has been located in this region, 45 minutes after training. However, as reported in this thesis (Chapter 3), an increase in fucokinase activity was found in the right roof 6 hours after training. The roof region contains the hyperstriatum ventrale (HV), a region which has often been cited as being important in biochemical and morphological correlates of training (see Chapter 2).

It would seem, therefore, that the increase in fucose incorporation observed after training in vivo (Rose & Harding 1984) and in vitro as reported in this thesis, is dependent on protein synthesis de-novo, and is not an independent training related biochemical process. However, this is still a very tenuous assumption, as one can never be certain that the CX treatment, in addition to, or instead of inhibiting protein synthesis, was not causing some other metabolic disturbance critical to training, (i.e. an accumulation of intracellular amino-acids, as reported by Hambley & Rogers (1979).

Still not totally convinced of the involvement of fucosylation of de novo protein synthesis in learning and memory, I decided to perform some experiments with tunicamycin, which selectively inhibits the process of glycosylation. Initially, I investigated the effect of

tunicamycin on fucosylation in vitro , with the ultimate aim to determine a concentration which would selectively inhibit fucosylation in vivo , and thus to examine its behavioural effects.

Tunicamycin is a nucleoside antibiotic, produced by Streptomyces lysosuperificus, which acts at the first step of the lipid-linked oligosaccharide pathway in the process of protein glycosylation. It inhibits the transfer of N-acetyl-glucose-1-phosphate from UDP-N-acetylglucosamine to dolichol phosphate to form N-acetyl dolichol pyrophosphoryl dolichol, the specific lipid moiety which serves as an anchor to the growing saccharide chain (Heifetz et al 1979). (The process of glycosylation is described in detail Chapter 2). In my experiments I used a range of tunicamycin concentrations, as it has been documented that the amount of antibiotic required for 50% inhibition varies greatly from one cell type to another and may range from 0.1 to 5.0 ug/ml (Elbien 1981). I also tested for any effect on protein synthesis, as some impure tunicamycin samples have been found to inhibit this process (Duskin & Mahoney 1982). The data show that very high concentrations were required to cause a 30 % inhibition in fucosylation in chick forebrain slices, but that protein synthesis was inhibited to the same degree. This is in keeping with the proposal that there is a regulatory link between glycosylation and protein synthesis, such that when glycosylation is blocked the synthesis of the protein portion of the glycoprotein is also prevented (Schwaiger & Tanner 1979). Tunicamycin is not a single compound but a mixture of homologous antibiotics, each of which contains one mole of fatty-acid with different carbon chain lengths (Takatsuk et al 1977). It is only by quantitative separation of the 10 tunicamycin homologues, by high performance liquid chromatography, that the specific homologue A1 (which exhibits very little inhibition of protein synthesis, but fully inhibits protein glycosylation) can be

obtained (Duskin & Mahoney 1982). Unfortunately, I was not equipped to perform such a separation. However, if a glycosylation inhibitor does cause a concomitant inhibition in protein synthesis (by a negative feed-back process), then its use would not have answered my initial question, due to the need to examine the inhibition of fucosylation alone. There is now a great deal of interest in the importance of protein glycosylation with regard to specific cell-cell adhesion molecules, and researchers have realized the importance of obtaining inhibitors which block the addition of specific sugars to the glycoprotein. These inhibitors would probably be directed towards the inhibition of the specific glycosyl-transferase, which transfers the sugar from the nucleotide-sugar complex to the growing glycan chain, such that the importance of specific sugars in glycoprotein function can be assessed. Thus, to determine the biological role of the terminal fucose moiety one would need an agent to selectively inhibit fucosyl-transferase activity.

It would seem from these experiments that the increase in fucosylation observed 30 min after training is a process which is coupled to an increase in protein synthesis as opposed to an increased fucosylation of proteins already present. One can only make this as a suggestion rather than an established fact, as protein synthesis was not 100% inhibited by CX. That CX caused a significant inhibition in protein synthesis in the CX treated over the S treated chicks, with a subsequent inhibition in fucosylation does strongly indicate that the two processes, protein synthesis de novo and fucosylation are linked. This conclusion enables me to reject the hypothesis that the observed increase in fucosylation after training is an independent, training-related, post-translational modification occurring on pre-existing proteins. Therefore, an increase in protein synthesis is occurring in the early stages of memory formation, as early as 30

minutes after training. This is supported by the data of Mark & Watts (1971), in which an intraventricular injection of CX, 10 minutes before but not 20 minutes after training chicks on a passive avoidance task, caused amnesia for the task. These workers suggest that an increase in protein synthesis begins very soon, probably within a few minutes of learning. However as inhibition of protein synthesis before training only leads to amnesia for the task as late as 1 hour after training, I would suggest that it is an important cell-biological mechanism of long-term memory, and not a necessary process for the establishment of the initial stages of memory formation (short-term memory; lasting up to 10 minutes, labile-term memory; lasting up to 30 minutes after training) (Gibbs & Ng 1977).

These conclusions agree favorably with the long established view that protein synthesis is involved in the formation of long-term memory (reviewed by Roberts & Flexner 1969; Rainbow 1979; Squire & Davis 1981; Davis & Squire 1984).

In summary, these experiments indicate that the increase in fucosylation observed after training is occurring on protein synthesis de novo, and is not a post-translational effect. This suggests that the long established view that protein synthesis is involved in learning and memory formation should be revised to include the observed increase in glycoprotein synthesis, and in particular, an increase in fucose-containing glycoproteins.

CHAPTER 6

GENERAL DISCUSSION

The aims of the work reported in this thesis were to provide further information on the biochemical correlates of learning and memory in the chick. I did not wish to add yet another behavioural-biochemical study to the literature archives; rather, I intended to expand on an already established research program, that of a specific biochemical correlate of training chicks on a one trial passive avoidance paradigm.

As Mark (1979) said; 'When the biochemistry of learning is mapped out, it will be as complex as that of the metabolic pathways of carbohydrate metabolism. Perhaps we have almost as great a quantity of information on learning, scattered throughout the literature, all we lack is the knowledge of the equivalent T.C.A. cycle'.

In Chapter 1, I have briefly reviewed the psychological classifications of learning and memory, and then considered in greater detail these processes from a physiological aspect. Although psychology and physiology are often regarded as two distinct disciplines, a basic knowledge of both is necessary before one can study the biochemistry associated with a behavioural paradigm. If the resulting biochemical data are to be rigorously assessed and attributed to a specific behaviour pattern, then an explanation of the

type of behaviour must be available; the biochemist must have a basic knowledge of the behavioural discipline. The greatest criticism which has been levelled at such researchers is their lack of care in choosing an experimental animal, and more specifically, in choosing an appropriate training task. It is reasonable to assume that if one is looking for biochemical changes associated with learning and memory, at the cellular level, one should choose an animal and training task that is particularly salient and relevant with respect to survival and reproduction (Domjan & Galef 1977).

The experiments reported in this thesis are based on training chicks on a one-trial passive avoidance task, a paradigm originally described by Cherkin (1969) and subsequently modified by Gibbs & Ng (1977). The chick is a good model for such neurobiological research because it is a precocial animal and has the ability to perform a large repertoire of behaviours at a very young age. In the passive avoidance paradigm, chicks are trained to peck a small bead. As chicks have a strong tendency to peck at small objects in their search for food, the training task is biologically relevant and relatively simple to perform, thus fulfilling the criterion of choosing a task that is part of the animal's normal behaviour. In the training trial, (M) trained chicks are presented with a methylanthranilate-coated bead and the (W) control a water-coated bead. On pecking of the first type of bead, the chick shows a characteristic aversive response and, on retest 10 minutes later, does not peck it. In the (W) control group, chicks will peck on initial presentation of the bead and on retest. One concludes that the (M) trained chick has learned to avoid pecking the bead while the (W) control has learned nothing. This behavioural process is readily seen in chicks in their natural environment; young chicks will peck and ingest many different small objects irrespective of their nutritive value; however, they learn very rapidly to

discriminate in one or two trials. Hogan (1973) gives the example of chicks showing clear signs of disgust when they peck at fresh droppings, their reaction is similar to that seen in the avoidance paradigm with methylanthranilate. Thus, the combination of animal and training paradigm used for experimental analysis in this thesis fit the criteria previously documented. Having established these important criteria the neuroscientist can proceed to investigate the physiological mechanisms underlying the training task in the quest to elucidate specific biochemical correlates.

The research group at La Trobe, led by Gibbs, have performed numerous studies with the young chick, their main interest lying in determining the temporal characteristics and classification of memory formation. On the other hand, Rose and his colleagues in The Open University, UK, have been concerned with the physiological mechanisms occurring in the brain as a result of training. As discussed in Chapter 2, research on the correlates of learning in the chick is diverse, including studies on receptor number, protein synthesis, and morphological and metabolic studies. My aim in this thesis was to investigate further, to increase the evidence of and confirm an already established biochemical change. This was the observed increase in fucose incorporation in vivo observed as early as thiry minutes after training and persisting for up to 24 hours (Burgoyne & Rose 1980a).

When a neurochemist sets out to determine what biochemical parameters change as a result of training, (s)he often has the preconceived idea that the change will ultimately have an effect (either transient or permanent) on the remodelling or restructuring of neurones, such that connectivity is altered; the ultimate outcome of such a change maybe expressed, as a morphological change in the neural

network. This hypothesis dates back as far as Hebb (1949) and Cajal (1911), and although there is evidence for biochemical and morphological changes associated with learning available throughout the literature, hard evidence indicating there is a direct relationship between these two processes is non-existent. With this hypothesis in mind, I decided to conduct further research on changes in glycoprotein metabolism, looking specifically at training-related changes in fucosylation. The importance of glycoproteins in neural membrane structure and function has been documented in Chapter 2. These molecules are excellent candidates as causal agents of neuronal morphological change due to their strategic position; this ability to cause change may be related to the type and composition of the glycan moiety, which confers glycoprotein specificity and heterogeneity. As fucose forms the terminal sugar of glycoproteins, it is an excellent probe to use in an investigation of changes in glycoprotein metabolism.

My initial experiments, reported in Chapter 3, were concerned with the investigation of the mechanism of the observed increase in fucose incorporation after training. I focused my attention on the activity of fucokinase, one of the enzymes involved in the metabolic pathway of fucose incorporation into glycoproteins. Preliminary experiments investigating the characteristics of fucokinase activity showed that activity was developmentally regulated; increasing activity was evident until day 20 post-hatch and thereafter, activity remained constant. It is well documented that the major maturational processes of synaptogenesis occur at day 7 in the chick (Dolezelova et al 1974). This finding suggested that fucokinase activity might be associated with processes other than those of neural development. On analysis of the optimal ionic conditions of fucokinase activity, experiments indicated that high concentrations of calcium inhibited

activity (Ic_{50} Ca^{2+} being 50 uM), and that a divalent cation, either Mn^{2+} or Mg^{2+}, was required for maximal activity. The divalent cation Mg^{2+} at a concentration of 3.3mM was the most effective. The conclusions drawn from this study indicated that fucokinase activity was maximal with low (uM) concentrations of Ca^{2+} and a concentration of 3.3mM Mg^{2+}.

My next series of experiments were concerned with the effect of training on fucokinase activity. The test-trial interval initially tested was 1 hour, which is the time at which a significant increase in fucose incorporation in vivo was detected (Burgoyne & Rose 1980a, Sukumar et al 1980). Lossner & Rose (1984) found that there was a significant increase in fucokinase activity in the right forebrain base at this time and my experiments attempted to replicate this effect and investigate the time course of this training- related increase. My results show that there was a significant increase in fucokinase activity in the left base 1 hour after training, and a further increase in activity in the right roof 6 hr after training in the (M) trained chicks compared with (W) controls. No increase was apparent at 10 hr and 24 hr after training. These results strongly indicate that the increase in fucose incorporation in vivo observed after training may indeed be related to an increased fucokinase activity. That a training-related increase was found initially in the left base and then in the right roof indicates that the effect is lateralized and agrees well with other lateralized biochemical effects associated with training, as discussed in Chapter 3. If the increase in fucokinase activity is related to an the observed increase in fucose incorporation in vivo as a result of training, what is the mechanism by which this increase is produced? The experiments reported in Chapter 3 indicate that fucokinase activity is not regulated by increases in the intracellular concentrations of calcium.

There is mounting evidence that an increase in intracellular calcium forms part of the physiological mechanism of learning and memory. In the hypothesis of Lynch & Baudry (1982) on the mechanism of long term potentiation (LTP) in the rat hippocampal slice preparation (discussed in Chapter 2), an increase in intracellular calcium plays an integral role. In the studies of Alkon (1984), who has extensively studied behavioural processes in Hermissenda, the physiological mechanism associated with conditioning is thought to be associated with prolonging the action potential spike by a reduction in the K^+ current. This change in K^+ conductance is thought to be associated with an influx in intracellular calcium. More specifically, Gibbs & Ng (1979) have shown that by increasing the extracellular concentration of Ca^{2+} in the chick, by an intracranial injection, the duration of short-term memory can be increased. Other research has shown that the activation of specific protein kinases by the Ca^{2+}-calmodulin complex, which also involves an increase in intracellular Ca^{2+}, may be associated with the mechanism of memory formation. This mechanism of kinase activation has been extensively studied in Routtenberg's laboratory. These workers have found that after training rats on a step-down avoidance task, there is an increase in phosphorylation of a specific protein called F1 in the trained compared to the control rats. This phosphorylation process is modulated by a Ca^{2+}-calmodulin protein kinase activation (reviewed by Greengard 1978). Rose & Tillson in unpublished work, have found that as a result of training chicks on a passive avoidance paradigm, there is an increase in calmodulin content in the forebrain of (M) trained chicks compared to (W) controls. This may indicate that activation of fucokinase activity is mediated by Ca^{2+}-calmodulin complex activation, with a subsequent increase in fucose incorporation after training. Evidence has been presented

which indicates that fucokinase is not regulated by increasing concentrations of calcium *in vitro*; the actual mechanism of stimulation *in vivo* can only be speculated. That an increased fucokinase activity may be associated with the observed increase in fucose incorporation *in vivo* as a result of training is evident. That the increase in fucose incorporation *in vivo* persisted for up to 24 hr, yet an increase in fucokinase activity was only detected up to 6 hr after training, may indicate that activation of another enzyme in the metabolic pathway is involved at later times. This work has not been investigated by any research group to date.

My attention was subsequently directed from the mechanism of the increase in fucose incorporation observed after training, to an investigation of the specific proteins involved in the increased fucosylation. This issue was previously addressed by Burgoyne & Rose (1980a), who analysed fucose incorporation *in vivo* into specific brain proteins after training. Their efforts to determine the molecular weights of specifically labelled glycoproteins were thwarted by the inability to obtain sufficient concentrations of radioactive label in the brain after an intraventricular injection. (This is a well documented problem with such *in vivo* work, as discussed in Chapter 4). Therefore, stimulated by the work of Schliebs *et al* (1985), in which an active *in vitro* protein synthesizing system was established in chick forebrain slices, I decided to determine if an active fucosylation system could be established *in vitro* in forebrain slices. Chapter 4 outlines preliminary experiments to find the optimal conditions for maximum fucose incorporation rates in chick forebrain slices; values as high as 37 nmol fucose/mg protein/ hr were recorded. This indicated that an active fucosylation system could be established *in vitro*. An electron microscopic study was undertaken to determine tissue integrity before and after a 3 hr incubation; the

results indicate that the tissue was in a good state of preservation. Subsequently, chicks were trained and slices from (M) trained and (W) control chicks were incubated in a medium containing radioactive fucose, and fucose incorporation rates were measured. The results show that 30 minutes after training, the significant increase, 15% ($P<0.05$), in fucose incorporation observed *in vivo*, was replicated *in vitro*, and that the increase was located in the right forebrain base. That the increase in fucosylation was located in this region agrees well with the results of Lossner & Rose (1984), who found a significant increase, 15%,($P<0.05$), in fucokinase activity in trained chicks compared to controls. This localization of increase to the forebrain base strengthens the argument for the involvement of specific nuclei located in this region; the paleostriatum augmentatum (PA) and the lobus parolofactorius (LPO) (Kossut & Rose 1984). A recent metabolic study by Rose & Csillag (submitted), using the 2-deoxy glucose method, has indicated that there is an increased uptake of this precursor into the right LPO of trained chicks compared to controls, as discussed in Chapter 4.

A study to determine fucose incorporation *in vitro*, 24hr after training, indicated that there was no training-related increase in (M) trained compared to (W) control chicks.

On further analysis to determine the specific subcellular fraction in which this increase (observed 1hr after training), was located, a significant increase in fucose incorporation was found in the P3 fraction. That this was the microsomal fraction was confirmed by electron microscopy. The ultimate aim of developing this *in vitro* method was to determine the proteins into which the increased fucosylation was occurring using SDS PAGE (polyacrylamide gel electrophoresis). To ensure that detectable quantities of radioactive

fucose would be incorporated into glycoproteins for this analysis, slices were incubated in media containing 100 times the specific radioactivity of ^{14}C-fucose that was normally used. On densitometric analysis of the stained gels, there was no difference in the absorbance of any specific protein band in the (M) trained and (W) control samples. However, on analysis of the quantity of labelled fucose in specific gel fractions, an increase in radioactive fucose was found specifically in a protein of 100K molecular weight, and there was a general increase in labelling of proteins with molecular weight 120K - 82K of trained chicks. This increase in labelling was as high as 40% in the 100K mol wt protein in the (M) trained compared to the (W) control sample, and this increased labelling was found in two out of three separate experiments. This is the first time that a training-related increase in the fucosylation of a protein of this molecular weight and range has been described, and the first report of an increased labelling of specific proteins in chick forebrain after training on a passive avoidance task. Murakami et al (In Press) in a study of the glycoprotein composition of post-synaptic densities (PSD) isolated from day-old chick brain, found that they contain at least 14 concavalin A binding glycoproteins of high, > 85K MW. The three most predominant glycoproteins were 170K, 180K and 125K. That training resulted in an increase in fucosylation of proteins in the molecular weight range 120K - 82K may indicate that these proteins include (or are the same as) the specific neural cell-adhesion protein, N-CAM. This molecule, which has received much research attention, was originally investigated by Edelman (1984), and is now confirmed to be identical to the protein named D2-CAM (Jorgensen et al 1980) and BSP-2 (Hirn et al 1983). N-CAM is thought to mediate cell-cell interactions which underlie the formation of complex neuronal pathways. This molecule is composed of three distinct polypeptides, each of which is

coded by a distinct gene, named A (mol wt 195K), B (mol wt 137K) and C (115K). The A and B polypeptides are integral membrane-associated proteins, whereas the C polypeptide can either be membrane-associated or secreted. These polypeptides are N-linked glycosylated and can be effectively labelled with fucose and sialic acid (Bock 1985). The interesting point, however, is that in the embryonic chick, these three polypeptides occur as a diffuse band of proteins, with molecular weights in the range 250K - 180K, when brain tissue is subjected to SDS PAGE (Rothbard et al 1982). It is only in the adult tissue that the three distinct polypeptides are observed as three separate bands on gel electrophoresis. Perhaps the increased fucosylation of proteins (120K - 82K), observed in this study, in a diffuse protein range, may be evidence that fucosylation is occurring on N-CAM. Further evidence which supports this theory is that N-CAM is a cell surface glycoprotein and it can be specifically labelled with fucose (Bock 1985). It has been shown to mediate adhesion between cells that express it, including muscle and glial cells in addition to neurones. More specifically, the adhesion or binding properties between N-CAM on different cells appears to involve the homophilic interaction between the molecule's carbohydrate moiety, such that modulation of the glycan moiety would lead to different binding properties. During development, N-CAM is modulated by the removal of sialic-acid residues, giving it different binding properties, and it is thought that this modulation is associated with the initial establishment of axonal pathways and projections (Rutishauser et al 1985). That fucose can specifically label N-CAM , where it forms the terminal sugar of the glycan moiety, may indicate that changes in fucosylation lead to the formation of different cell-cell interactions. During development, cell-cell interactions are influenced by the sialilation of N-CAM; a glycosylation process, the degree of fucosylation of a

glycoprotein or indeed N-CAM also influences the formation of specific cell-cell interactions (Edelman 1984). If this was occurring, then the ability to form specific cell-cell interactions through a fucosylated glycoprotein could be the mechanism of neural plasticity and network remodelling; the physiological basis of learning and memory. This biochemical change leading to membrane modulation may explain the morphological changes observed after training chicks on PAL (Stewart et al 1984) as discussed in Chapter 2. These include, an increase in the length of the post-synaptic thickening in the (M) trained chicks compared to that in the (W) controls. Of course,this is only speculation and requires further, intensive research.

In an attempt to determine if the mechanism of increased fucosylation associated with training was elicited by a neurotransmitter action, the effect of a range of neurotransmitters added to the incubation medium was investigated. The transmitters were studied in a range of concentrations, but noradrenaline, dopamine, 5-hydroxy-tryptamine and acetylcholine did not cause an increase in fucosylation in chick forebrain slices. The concentration and distribution of transmitters in the avian chick brain have been extensively studied, and indicate that 5- hydroxytryptamine in concentrations of 0.70 ug/g, is the most prominent neurotransmitter, followed by noradrenaline (0.60 ug/g) and 5-hydroxy-tryptamine (0.029 ug/g) (reviewed by Juorio 1983). My study of the effects of acetylcholine on fucose incorporation in vitro was stimulated by the observation that, on training chicks on a passive avoidance task, there is a transient increase in muscarinic receptors in (M) trained compared to (W) control chicks (Longstaff & Rose 1980). I thought that this training related increase may be associated with an increase in acetylcholine concentration, which might also have an effect on fucose incorporation rate. However, my results do not provide

evidence for such a link and indicate that the training-related increase in fucose incorporation was not mediated by any of the neurotransmitters studied.

Having established that there was an increase in fucose incorporation *in vitro* as a result of training and that this increase was confined to proteins of specific molecular weight range 120K - 77K, I was interested in obtaining more information on these proteins. I wanted to determine if these proteins were synthesised *de novo* and subsequently fucosylated as a result of training, or if the training-related increase in fucosylation was an independent training effect occurring on pre-existing proteins. In order to conduct this investigation, as reported in Chapter 5, I used the protein synthesis inhibitor cycloheximide (CX). My initial experiments were concerned with determining the effect of CX on fucosylation *in vitro*. When 1mM CX (which inhibits protein synthesis by 95% in chick forebrain slices *in vitro*; see Schliebs *et al* (1985), was added to the incubation medium, fucosylation was only inhibited by 60% after a 3 hr incubation. This indicated that some fucosylation was occurring on pre-synthesised proteins. Subsequently, slices from (M) trained and (W) control chicks were incubated in the presence of CX; the training-related increase in fucosylation in the (M) trained chicks still persisted. At this stage my results supported the hypothesis that the increase in fucosylation due to training was indeed an effect independent of protein synthesis *de novo*. However, there was a delay of 45 min between training and slice incubation in the presence of CX, such that a training-related increase in protein synthesis could have occurred during this period; the observed increase in fucosylation could have occurred on these proteins. In my next experiment CX was injected into the chick brain 10 minutes before training so that protein synthesis would be inhibited. The concentration and route of

injection were similar to those used by Gibbs et al (1973), in which there was an 80% inhibition of protein synthesis in vivo. In order to determine the percentage of protein inhibition due to CX treatment over the S treated chicks (the sham control group), each chick was injected with ^{3}H- leucine 15 minutes after training. Slices from the right forebrain base (the region in which the training-related increase in fucose incorporation was found) were then prepared for incubation in ^{14}C-fucose for a period of 3 hr. After this the slices were analysed for leucine incorporation in vivo and fucose incorporation in vitro, in the four treatment groups; CX (M) trained, CX (W) controls, S (M) trained and S(W) controls. Although there was a 50% inhibition of protein synthesis in vivo due to CX treatment, there was no effect on fucosylation rates in vitro in slices from the CX treated chicks compared to slices from the S treated chicks. This may have been due to the CX been washed out of the tissue durning the 3 hr incubation, such that protein synthesis de novo was occurring. On analysis of fucosylation rates due to training, a 15% increase in fucose incorporation rate was found in the CX (M) trained chicks over the CX (W) controls. However, no such increase in fucosylation was observed in the S (M) trained chicks compared in the S (W) controls. That a training -related increase was still evident after CX treatment in vivo seemed to further indicate that protein synthesis de novo was not involved, but one could not be entirely satisfied with this conclusion as no such increase was found in the S (M) treated chicks compared to the S (W) controls. I cannot explain this result, but attribute it to the many confounding variables and thus often irreproducible nature of such biochemical-behavioural experiments, as discussed in Chapter 2. Because no training-related increase in fucosylation was found in the S treatment group, which was the sham 'control' group for the CX treated chicks, I cannot conclude any

satisfactory results from this experiment.

An experiment was then designed to investigate the effects of training on both leucine and fucose incorporation *in vivo* in CX and S treated chicks. On analysis of leucine incorporation, there was a significant 30% inhibition in protein synthesis due to CX treatment (in CX (M) compared to the S (M) and also in the CX (W) compared to the S (W) control). There was a significant inhibition in fucosylation in the CX treated chicks compared to the S controls, (40% inhibition was recorded when CX (W) was compared toS (W) and a 70% inhibition was recorded when the CX (M) was compared to S (M)). When I analysed the effects of training on fucose incorporation, I found there was no increase in fucosylation in any forebrain region in the CX (M) trained chicks when compared to the CX (W) controls. However, there were significant increases in fucosylation in all four forebrain regions in the S (M) trained compared to the S (W) controls. The results from this experiment indicate that when protein synthesis is inhibited before training by CX injection, the increase in fucosylation observed in (M) trained chicks compared to (W) controls is abolished. This suggests that the training-related increase in fucosylation, which has been clearly indicated in previous experiments *in vitro* as detailed in chapter 5 and *in vivo* by Burgoyne & Rose (1980), is occurring after protein synthesis *de novo*, as opposed to a protein post-translational effect. However, this conclusion is open to criticism as protein synthesis was not 100% inhibited by CX, or indeed inhibition of increased fucosylation may have been directly inhibited by the action of CX. These observations clearly indicate the sometimes ambiguous nature of interventitive experimentation.

Although evidence for the importance of protein synthesis in the formation of long-term memory is available in the literature, there are a number of reports which cast some doubt on this finding, in particular with regard as to whether the amnesia caused by protein synthesis inhibitors is transient or permanent. Flood & Jarvik (1976) and Davis et al (1978) suggest that memory may recover after cerebral protein synthesis inhibition if the initial inhibition of consolidation is incomplete. These workers found that anisomycin-induced amnesia for a passive avoidance task was reversed by pretest injection of amphetamine or re-exposure to the training apparatus; they presented evidence that recovery only occurred in mice that showed some memory of training in a pretest. Also, Gibbs (1976) found that amphetamine counteracted CX-induced amnesia when chicks were trained on a passive avoidance task, but only if it was administered while short-term, protein independent memory was still present. This suggests that amphetamine arrests the decline of memory at the time of injection. These experiments indicate that protein synthesis is not the only essential mechanism related to memory formation, and that the transient amnesia which is sometimes reported may be due to some side-effect of the inhibitor used. This is one of the major problems associated with the this type of interventive research, as discussed in detail in Chapter 5. Despite the obvious limitations of interventive methodology (as previously referred to), a number of well-designed experiments have been performed utilizing this approach, and these have permitted conclusions to be drawn about the relationship of brain macromolecular synthesis to behavioural processes (Gibbs & Ng 1976). Probably the most desirable way in which to conduct research is to combine both correlative and interventive methodology, such that they complement one another. This is the approach I have adopted in the experiments reported in this thesis,

first the correlative approach is taken, and then, I have confirmed my results using interventive methodology.

The conclusions from this set of experiments are in agreement with the long established view that protein synthesis is an essential mechanism for the establishment of long-term memory, and that although protein synthesis is occurring in the early stages of memory formation (as early as 30 minutes after training) its occurrence is not essential to show recall at this time after training (reviewed by Squire & Davies 1984). That protein synthesis is occurring in the early stages of memory formation but is only a necessary physiological mechanism for the establishment of long-term memory may provide evidence for the parallel model of memory formation as opposed to the sequential model, as discussed in detail in Chapter 1. This would indicate that immediately and shortly after training, a number of physiological mechanisms are simultaneously activated if long term-memory is to be established. Thirty minutes after training chicks on a passive avoidance paradigm, as reported in this thesis, the mechanisms of memory formation may include an increased neuronal activation resulting in increased fucokinase activity. Simultaneously, an increase in protein synthesis is activated with a consequent increase in fucosylation forming a membrane glycoprotein of molecular weight in the range 120K - 82K. With this increase in fucose-containing glycoproteins, there will be increased ability to form new homophilic glycoprotein interactions. As these glycoproteins are predominantly membrane-bound, the ability to form new interactions may be the mechanism of cell and neuronal network modulation underlying the process of neuronal plasticity. This may be the physiological basis of learning and memory formation.

Although each stage of memory formation is specifically associated with a physiological response, the occurrence of each response will depend on the initial strength of learning and neuronal activation. In this respect, therefore, the establishment of the later stages in memory formation are dependent on the establishment and strength of the initial stage, that of short-term memory. As such, the optimum model of memory formation would encompass the features of both a sequential model and a parallel model, as proposed by Kessner (1973).

In summary, the work reported in this thesis satisfies the aims of confirming and extrapolating a previously investigated biochemical correlate of learning and memory, i.e. an increase in fucose incorporation in vivo as a result of training, reported by Burgoyne & Rose (1980). The mechanism of this increase has been investigated by experiments on fucokinase activity; fucokinase is an enzyme in the metabolic pathway of fucose incorporation into glycoproteins. On training chicks, there is an increase in fucokinase activity which is localized to the left forebrain base and right roof 30 minutes and 6hr after training, respectively. These results suggest that the increase in fucose incorporation as a result of training may be related to increased fucokinase activity. I then successfully obtained an experimental system in which active fucosylation in vitro was established in chick forebrain slices. With this in vitro method, the training-related increase in fucosylation observed in vivo was replicated in vitro. When forebrain slices from (M) trained and (W) control chicks were incubated in a ^{14}C-fucose containing medium, a significant increase in fucose incorporation was detected in the right forebrain base of trained chicks compared to controls. With the advantages of an in vitro method, I was able to determine the molecular weight of a band of proteins into which this increased

fucosylation was occurring by gel electrophoresis. The labelled proteins were in the molecular weight range 120K - 82k. The possibility that changes in fucosylation (a terminal glycan sugar), may mediate changes in the homophilic interaction of such a membrane glycoprotein in the process of neural modulation and plasticity (learning and memory), is apparent. Finally, a series of experiments was conducted to determine if the training-related increase in fucose incorporation was an independent protein translational effect. The results from these experiments suggested that, consequent to training, there was an increase in protein synthesis de novo followed by fucosylation in the (M) trained chicks compared to the (W) controls.

In conclusion, the work reported in this thesis presents further evidence for the involvement of glycoprotein metabolism, in particular fucose-containing glycoprotein synthesis as part of the physiological mechanism of learning and memory in the chick.

CHAPTER 7

FUTURE DIRECTIONS

As with any research, I feel that I have created more loose ends and unanswered questions than I have provided answers! However, I believe that the most exciting result produced in this thesis is the observation of specifically labelled proteins, in the molecular weight range 120K - 81K and more specifically 100K, as a result of training. I think it would be worthwhile repeating these experiments and in particular to try and modify the in vitro system such that longer incubation times could be studied. If such a system was feasible then the training-related increase in fucosylation may be loacted in a synaptosomal fraction. If there was no restriction on length of incubation time, then maximum labelling of glycoproteins could be achieved, which could then be studied by gel electrophoresis. From the gel experiments reported in Chapter 4, it appears that there is selective labelling of glycoproteins within a specific molecular weight range. I think it is necessary to repeat these gel experiments using a lower acrylamide gel concentration, to get a greater separation of proteins within this molecular band. This might aid in the isolation of specific proteins which are fucosylated as a result of training. One could then preform some blotting experiments to identify the glycoproteins involved. If this was successful then the whole area of monoclonal antibody production against specific proteins

could be investigated. With these tools one could then investigate and confirm the existence of specific training-related proteins. In particular it would be interesting to preform some immunocytochemistry with N-CAM antibody, to determine if it is one of the glycoproteins specifically labelled as a result of training.

As a follow up to the experiments on fucokinase activity, it would be interesting to analyse further the possible mechanism of activation as a result of training. In order to conduct further experiments on the fucokinase enzyme, I would suggest that a different experimental assay than that reported in Chapter 3, might be used. Preferentially, I would try and set up a continuous assay system to monitor fucose-1-phosphate production, which would incorporate the measurement of reduced NADP2H by spectophotometric analysis. (This assay, a coupled-enzyme assay, is based on that for the measurement of glucose-1-phosphate). One could conduct a series of experiments to establish if in fact fucokinase was a Ca^{2+}-calmodulin activated kinase, and to determine more specifically if changes in intracellular calcium are associated with activation. A series of experiments to determine the effect on fucokinase activity of reducing intracellular Ca^{2+} levels could be studied by the addition to the medium of ethyelene glycol tetra-acetic acid (EGTA), a specific Ca^{2+} chelator. (Due care should be excercised to ensure that there is no change in pH levels of the incubation medium due to the acidic nature of EGTA). It would also be interesting to determine if fucokinase activation was effected by specific Ca^{2+}/Mg^{2+} ratios, as hypothesised in Chapter 3. Also, further experimentation on and analysis of the effect of training on fucokinase activity, in particular the analysis of Vmax and Km of the activated enzyme, would determine more specifically the mode of activation.

Another avenue of research would be the analysis of the other enzymes in the metabolic pathway of fucose incorporation into glycoproteins, in particular the effect of training on fucosyl-transferase activity. Such a study, incorporating a time course of activity after training, would further supplement the investigation reported in this thesis.

With the in vitro preparation, one could conduct a series of experiments to determine if changes in the intracellular concentration of calcium has any direct effect on fucose incorporation. One could use the calcium ionophore A23187 or, alternatively, use potassium depolarization to induce an increase in intracellular calcium, and monitor fucose incorporation in vitro. These experiments might provide specific evidence for a link between an increased fucokinase activity and an increase in fucose incorporation, associated with changes in intracellular calcium concentration, as a result of training, thus, leading to a clearer understanding of the effects on fucose metabolism when chicks are trained on a passive avoidance paradigm.

On the behavioural aspect, it would be interesting to study more specifically individual chick behaviour during the training paradigm. One could monitor in particular, the number of bead pecks each chick makes during presentation of each bead, rather than adopting the protocol of timimg each presentation as reported in this thesis. It would also be instructive to preform experimental analysis on all chicks, those that reached training criteria and those that did not. This would then give a clearer view of the specific correlates associated with the training procedure, as opposed to changes resulting from side-effects of training. To further consolidate the training-related changes as reported in this thesis, it would be

interesting to look at a different behavioural paradigm with the chick. One could determine if changes in fucose metabolism were also observed when chicks are imprinted or trained on a different task. This approach would also add evidence that specific biochemical changes were associated with the physiological mechanisms of learning and memory, and were not specific to a particular training paradigm.

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